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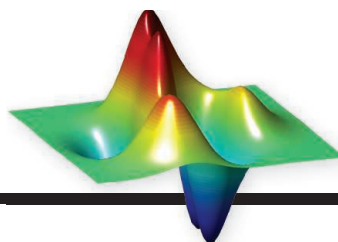
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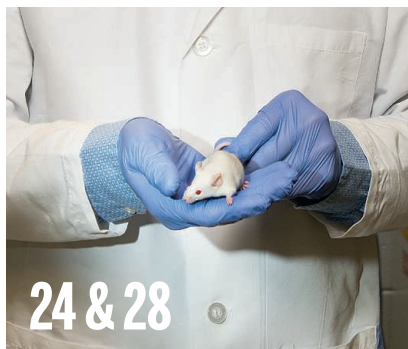
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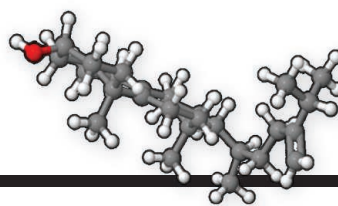
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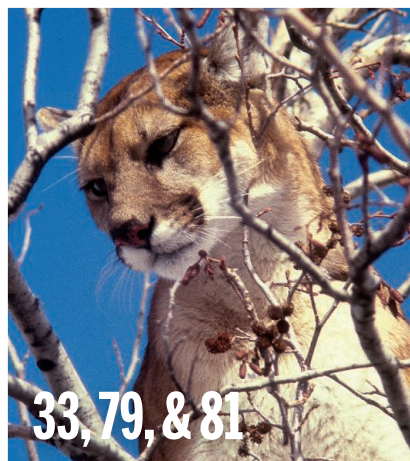
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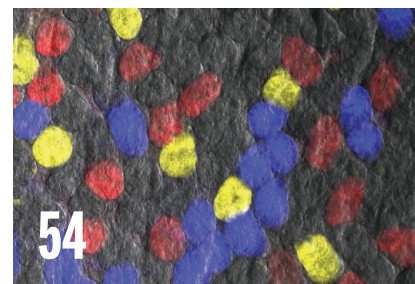
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ON THE COVER



To make mice better mirrors of human cancer, researchers are building “avatars” with the cancer of a particular patient, or engineering mice to spontaneously develop tumors just like people do. The work marks a sea change in cancer biology and is stirring hope that new mouse models will pave the way to more personalized care. See pages 24 and 28.
Photo: Joe McNally

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Building agricultural research

Nine billion people are expected to inhabit Planet Earth by 2050. Without agricultural research, there is little hope of sustaining this population surge, given that arable land and water supplies are fixed commodities. Yet for decades the agricultural sector has suffered from neglect. If we want to combat new strains of pests that destroy crops, find new crop varieties enriched in nutritional value, improve yields, develop resistance to disease and drought, and provide environmentally sensitive cultivation practices, then agricultural research must be a priority. Why isn't it?

In the 1970s, as a biology professor at Stanford University, I worked with the Office of Science and Technology Policy in the White House to discover what incentives might encourage the growth of competitive peer-reviewed agricultural research. At the time, other major federal agencies such as the U.S. National Institutes of Health were enjoying boosts in competitive research funding. On the other hand, the U.S. Department of Agriculture (USDA) used "formula" funding on a regional or commodity-focused basis, largely through the public land-grant universities. That process yielded key advances, increasing our ability to feed more people: improved fertilizers, artificial irrigation, harvest mechanization, and hybridization. But many researchers believed that advances in basic science would provide new ways to revolutionize agricultural production. We found it hard to understand why a brilliant cell biologist had to seek support from another agency to fund innovative research, rather than make a major contribution to how we grow food through support from USDA. A modest competitive grant program was launched then, but its survival in future budget cycles turned out to be perilous.

What happened? Over the past 35 years, new ventures in U.S. public investment in agriculture research and development confronted a steady decline. At the same time, great advances in biochemistry, cell and molecular biology, and genetics were being made through increased funding to other agencies for competitive merit-based

research grants. Because of the earlier history, agricultural research is now in a deficit position with respect to the infrastructure, human capital, and policies needed to address the challenges of food security.

A real revolution in agricultural research is possible if today's deeper knowledge, new tools, and advanced capacities could be effectively blended. Fortunately, in response to a USDA task force (headed by William Danforth, then the chancellor of Washington University), Congress created the National Institute of Food and Agriculture (NIFA) within USDA in 2006 as a means to modernize the management of fundamental agricultural research. NIFA

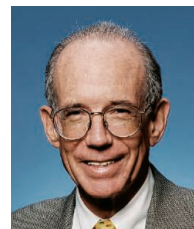
now manages \$200 million in competitive merit-based grants for fundamental agricultural research through its Agriculture and Food Research Initiative. That new agency is one of the rare federal research programs to have shown steady increases over the past 5 years, making this a major turnaround in competitive research support.

Despite this success, the current level of funding for USDA falls short of the opportunity presented by the agricultural sciences. Certainly, today's fiscal climate makes it hard to argue for extending discretionary federal spending. That is why non-partisan science-based groups that have seen the need to bolster research in agriculture and are willing to work for its improvement are important players. One is the recently created organization called Supporters of Agriculture Research (SoAR). William Danforth, appropriately,

is its chairman. SoAR includes eminent scientists across disciplines as well as representatives of consumer and commodity groups, and I am eager to work with them. High on SoAR's agenda is to increase funding for competitive grants, so that USDA can encourage interdisciplinary and innovative research.

The much-needed revolutions in agriculture can only come about through the investments that we make now. Nine billion people will, we hope, reap the benefits of today's wise decisions.

– Donald Kennedy



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"...nonpartisan science-based groups that have seen the need to bolster research in agriculture...are important players."

IN BRIEF

Fatal eruption at Mount Ontake

Smoke rises from the volcano, hours after its 27 September eruption.



Japan's Mount Ontake erupted without warning on 27 September, catching hundreds of climbers by surprise at lunchtime. The explosion killed more than 30 hikers near the peak and blanketed the mountain with ash. The Japan Meteorological Agency recorded seismic activity beneath the mountain in September, including 85 tremors on 11 September alone. But there was no noticeable crustal deformation and the seismic activity died down, so the agency did not raise its alert above 1 on the 1-to-5 volcanic warning scale. Yasuyuki Miyake, professor of volcanology at Shinshu University in Matsumoto, says it was a phreatic eruption, driven by steam heated by magma deep underground. Unlike magma eruptions, typically presaged by crustal deformations, "phreatic eruptions occur unexpectedly and are very difficult to predict," he says.

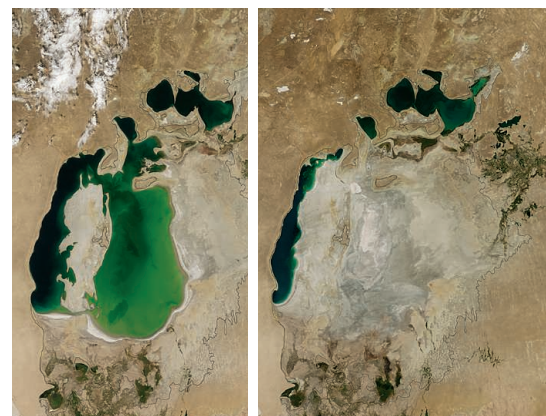
AROUND THE WORLD

Sea change for Chinese academy

BEIJING | A massive overhaul is in the offing for the Chinese Academy of Sciences (CAS), China's biggest research institution. Calling research at CAS's 104 institutes "fragmented and inefficient," academy President Bai Chunli has launched a structural reform that will merge some institutes in the name of producing more innovative and weighty results. One of the big winners is the National Space Science Center (NSSC), which will merge with two CAS divisions to form an Innovation Academy in Space Science, akin to a NASA research center, with staff expected to triple to 3000 by 2030, says NSSC Director General Wu Ji. Some researchers may view the reform "as a top-down effort that eventually will fall short of reaching its goal," says Poo Mu-ming, director of CAS's Institute of Neuroscience in Shanghai. He, however, calls it "inevitable and timely."

The vanishing lake

ARAL SEA | Now you see it ... now you don't. To irrigate cotton fields in Central Asia, Soviet authorities in the early days of the Cold War diverted water from the two major tributaries of the Aral Sea, once the world's fourth largest lake. In 2005, Kazakhstan built a dam to save the small Northern Aral Sea; by then the Southern Aral had fissured into eastern and western lobes. Images from NASA's Terra satellite



Aral Sea in 2000 (left) and 2014.

PHOTOS: (LEFT TO RIGHT) AP PHOTO/KYODO NEWS; NASA EARTH OBSERVATORY (2)

Downloaded from www.sciencemag.org on October 9, 2014



The fringe on the backs of modern myrmecophilous beetles secretes a substance that ants love.

Even early on, beetle made itself at home among ants

Ants don't usually welcome strangers into their nests. But myrmecophilous beetles are the exception, having evolved special brushes on their backs that secrete a substance that the ants adore. This love potion makes an ant treat this lifelong intruder like kin, even feeding it mouth to mouth. The beetles are quite specialized obligate social parasites (meaning they need the ants to survive), with a much reduced, nonsegmented abdomen and a head that's basically a straw. The relationship dates back at least 52 million years, report Joseph Parker of Columbia University and David Grimaldi of the American Museum of Natural History this week in *Current Biology*. They describe finding a fossil beetle trapped in amber from India that appears to be a missing link; it looks a lot like modern myrmecophilous beetles, suggesting the ant-beetle affair was in full swing well before ants became as plentiful as they are today. The fossil may represent the oldest known example of social parasitism. <http://scim.ag/fossilbeetle>

document the eastern lobe drying up completely this summer—due to a combination of decreasing precipitation, vanishing snowpack, and irrigation withdrawals—for the first time in modern history.

New rules for risky studies

WASHINGTON, D.C. | Academic scientists with U.S. government funding who work with any of 15 dangerous microbes or toxins will soon have to flag specific studies that could potentially be used to cause harm and work with their institutions to reduce risks, according to new federal rules. The long-awaited final rules will require that scientists using the 15 agents in seven types of experiments that represent so-called dual use research of concern (DURC)—such as making an agent more transmissible or resistant to drugs—notify a special review committee within their institution. If this

committee agrees the research is DURC, the institution must notify the funding agency and develop a risk mitigation plan. Some onlookers said that the rule lacks teeth and should cover more pathogens. <http://scim.ag/DURCRules>

Climate change led to heat waves

WASHINGTON, D.C. | Australia's record-setting heat wave in 2013 is directly linked to human-caused climate change, report five separate studies published this week in the *Bulletin of the American Meteorological Society*. The five papers were part of a special issue of the journal, *Explaining Extreme Events of 2013*, which included 22 studies focusing on 16 different extreme weather events, including the California droughts and Colorado's extreme rains. But the studies found that any link between climate change and other extreme events,

including droughts, floods, and storms, was murky. "[Attribution science] is hard, cutting-edge research," said Stephanie Herring, the report's lead editor and a researcher at the U.S. National Climatic Data Center in Boulder, Colorado. <http://scim.ag/extremeclimate>

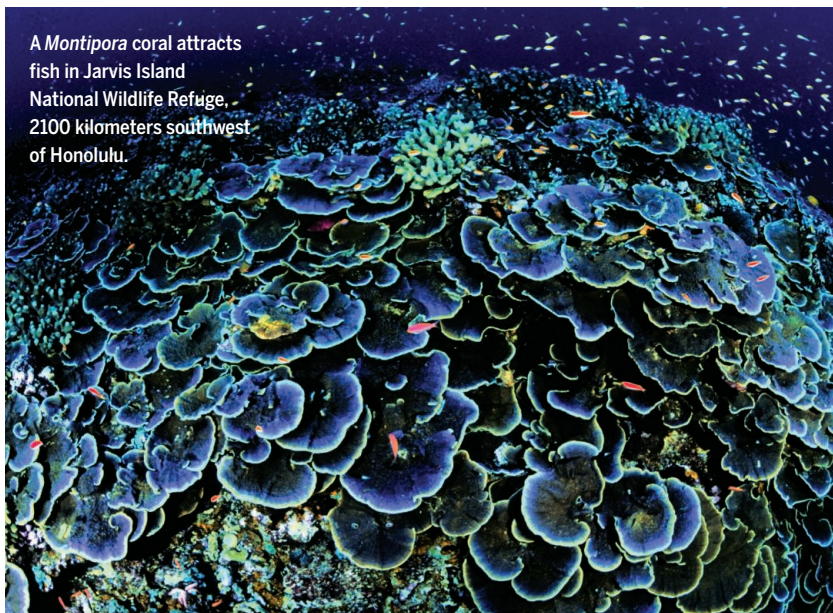
New Ebola vaccine test stalled

AMES, IOWA | Researchers eager to test an Ebola vaccine are frustrated by what they call "needless" delays. "[We] could be doing something, but things are not moving forward," virologist Stephan Becker of the University of Marburg in Germany told *Science* on 29 September. He is part of a consortium ready to conduct a safety trial of a candidate vaccine licensed to NewLink Genetics of Ames, Iowa. The vaccine—which contains an Ebola gene stitched into a livestock pathogen known as vesicular stomatitis virus—was developed by the Public Health Agency of Canada in Winnipeg. The company says issues including insurance and intellectual property concerns are slowing the process. Meanwhile, testing of other vaccines is moving ahead, and researchers and others met at the World Health Organization in Geneva, Switzerland, this week to discuss how to accelerate the process. <http://scim.ag/Ebolastall>

UNESCO meeting under fire

PARIS | The United Nations Educational, Scientific and Cultural Organization (UNESCO) is potentially wading into hot water on 8 October when it hosts a meeting in Paris set up by Nobelist and HIV co-discoverer Luc Montagnier to discuss his controversial research on what has become known as "the memory of water." The afternoon will feature talks about the theory—endorsed by Montagnier but widely ridiculed as pseudoscience—that water can carry information via an electromagnetic imprint from DNA and other molecules. John Crowley, head of UNESCO's Research, Policy and Foresight Section, says the agency doesn't endorse or oppose Montagnier's ideas but offers an "intellectual space" to discuss them, a "normal procedure" because Montagnier chairs a foundation associated with UNESCO and is housed at the agency's headquarters. Andy Lewis, who hosts the blog *The Quackometer*, says UNESCO is conferring credibility on the research, which some see as scientific support for homeopathy. Montagnier, however, says homeopathy is not on the meeting's agenda. <http://scim.ag/watermemory>

A *Montipora* coral attracts fish in Jarvis Island National Wildlife Refuge, 2100 kilometers southwest of Honolulu.



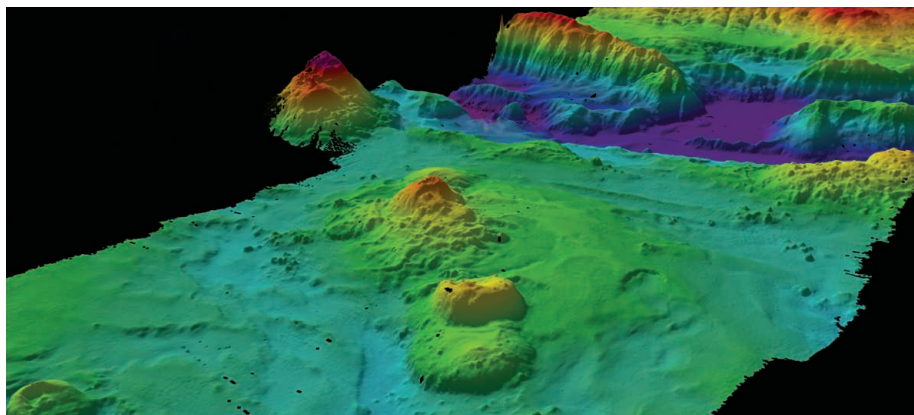
U.S. creates marine megareserve

President Barack Obama has moved forward with a plan to expand U.S. marine reserves in the remote central Pacific Ocean into a massive national monument. On 25 September, Obama signed a proclamation expanding the Pacific Remote Islands Marine National Monument to about 1.27 million square kilometers, up from about 225,000 square kilometers. This past June, White House officials said they were considering banning fishing and imposing other protections in an even bigger area. But they pulled back, in part as a result of opposition from the relatively small U.S. tuna fleet, which takes up to 4% of its catch in the region. Still, marine conservation groups were pleased, calling the expansion a major step. <http://scim.ag/USmarineres>

Seabed mapped in MH370 search

SOUTHERN INDIAN OCEAN | The ongoing hunt for missing Malaysia Airlines Flight 370, which disappeared on 8 March, has yet to locate any remnants of the plane. But it is producing a first look at some previously unknown seafloor features. A team from the Australian Transport Safety Bureau (ATSB), which is leading the search, has been using multibeam sonar to scan 60,000 square kilometers of the “priority” search area, a long narrow arc in the southern Indian Ocean. On 24 September, ATSB released the first detailed images, revealing features such as extinct submarine volcanoes 1.5 kilometers high and depressions 1400 meters deep. When the mapping is complete, the searchers plan to deploy deep-sea vehicles to look for wreckage.

The search area in the Indian Ocean is a dramatic landscape of extinct seamounts and deep depressions.



Plasma's role in deadly battle

LAUREL, MARYLAND | In 2002, during the War in Afghanistan, a U.S. Chinook helicopter was accidentally ordered to land atop snowy Takur Ghar mountain, which was controlled by heavily armed al-Qaida forces. Satellite radio operators tried to warn the pilots of the danger, but the message never got through. Now, researchers from the Johns Hopkins University Applied Physics Laboratory say that “plasma bubbles” may have contributed to the communications blackout. The bubbles occur in the ionosphere when ionized gas, or plasma, at lower altitudes loses density and rises through heavier plasma above. That turbulence can cause radio waves to refract, leading to signal loss. Using UV imaging data from a NASA satellite, the scientists reconstructed the ionosphere above the battlefield and confirmed the presence of a bubble, they reported online last month in *Space Weather*. <http://scim.ag/plasmabubbles>

NEWSMAKERS

Getting to Mars, without stress



India on 23 September became the first nation to reach Mars on its first try, putting its \$70 million Mars Orbiter (called Mangalyaan in Hindi) into orbit (http://scim.ag/Mars_India). Avionics engineer

K. Radhakrishnan, 65, chair of the Indian Space Research Organisation, discussed the feat and what's next.

Q: How challenging was it to develop Mangalyaan?

A: Time was of the essence. We had to schedule [the launch] for November 2013, and we started with a feasibility study in August 2010. So in less than 2 years we needed to realize a spacecraft worthy of flying to Mars, going through all the tests and simulations.

Q: What's next?

A: We could look for another good scientific mission. We also have Chandrayaan-2, which is going to be a [moon] lander and rover [scheduled for launch in 2016 or 2017]. There will be instruments on both for making in situ observations.

Q: You are an engineer, but you also sing and practice Kathakali, a stylized dance. How do you find time?

A: Half an hour in a day is not [a lot] for doing something that will relax you, take all your stresses out.

PHOTOS: (CLOCKWISE FROM TOP) JIM E. MARAGOS/USFWS; PALLAVA BAGLA, AUSTRALIAN GOVERNMENT/AUSTRALIAN TRANSPORT SAFETY BUREAU

IN DEPTH



INFECTIOUS DISEASES

When Ebola protection fails

Repeated cases among health care workers are a puzzle, but more staff and better training may lower risks

By Jon Cohen

In July, as Ebola was exploding in Liberia, Senga Omeonga worked as a doctor at St. Joseph's Catholic Hospital in Monrovia. Among the patients he cared for was the hospital's director, who had diarrhea and was vomiting repeatedly, but tested negative for the Ebola virus. "I was exposed to that patient day by day," says Omeonga, who originally is from the Democratic Republic of the Congo. When the director didn't respond to treatment, a second test was done, which came back positive. That was 10 days after his first test.

Omeonga wore what he calls "light" personal protective equipment (PPE) after he learned the man was infected, which included a surgical gown as opposed to a heavy plastic apron, gloves that he thought were too short, and a face shield and mask. By the last few days of the patient's life, he says, the staff was keeping its distance. "Everyone was afraid to touch him," Omeonga says. "He was screaming. I removed his nasogastric tube and he was fighting." On 2 August, the hospital director died and Omeonga himself came down with Ebola.

Omeonga, along with two other health care workers infected in Liberia, Kent Brantly and Nancy Writebol, has received widespread media attention for receiving an

experimental cocktail of antibodies called ZMapp. All three survived; none of them knows if the treatment helped. But all three wonder about another question that has important implications for other health care workers: How did they become infected?

Surprisingly, no one has a firm answer. "Every day I'm still thinking, When was I contaminated?" Omeonga says, although he suspects the hospital director was the source. Writebol, a clinical nurse associate who worked for a missionary group called SIM at the ELWA 2 Ebola Treatment Center in Monrovia and helped health care workers don and doff PPEs, is similarly stumped. "Nobody is really sure, least of all me," she says. Brantly, a doctor in the same center, also has only hunches but says, "I am fully convinced that I did not contract Ebola in my work in the treatment unit." (Read Q&As with Ebola survivors at <http://scim.ag/ebola14>.)

As of 23 September, the outbreak had sickened 375 health care workers and killed 211, according to the World Health Organization. A clearer understanding of the risks could lead to better precautions and ease the minds of those thinking of joining the fight. But few studies have analyzed the relative risks of blood, urine, vomit, and other bodily fluids that health care workers encounter. And doctors and nurses rarely can pinpoint risky lapses in their behavior, says epidemi-

Nancy Writebol helped health care workers in Liberia don protective equipment before they entered an Ebola treatment unit.

ologist Daniel Bausch of Tulane University in New Orleans, Louisiana, who worked in Ebola units in Guinea and Sierra Leone when this outbreak surfaced.

"Very few people have anything specific to say," Bausch says, although many, like Brantly, doubt that they got infected in the Ebola unit itself, where precautions are most stringent. "There's a tendency to want to believe people get infected outside the ward because it makes us feel better. It's probably a mixed bag."

Brantly, Writebol, and Omeonga say they had ample training about how to protect themselves. "Our process was very safe," says Brantly, who worked for the Christian relief group Samaritan's Purse. "It is my opinion that during an Ebola outbreak, the safest health care job is working in the Ebola treatment unit." The hidden danger, they say, lies in patients whose status isn't known.

Brantly suspects he was infected while working in the emergency room, outside the treatment unit, and saw a patient who was diagnosed with Ebola only after she died. He wasn't wearing PPE at the time. "It is in clinics and emergency rooms and hospitals where you have to look at every patient and ask yourself 'Should I be concerned that this patient might have Ebola?'" he says. But it's a risk that is, in practicality, impossible to eliminate.

Omeonga, too, says new patients present a serious risk. "A lot of them were lying when they came to the hospital," he says. "They didn't even tell you they're having fevers. They'd say they fell down or were on a motorbike or someone pushed them or they went to work and passed out." He was one of 15 who became infected at his hospital, presumably all by the ailing director. Nine of them died. The hospital closed.

When helping staffers doff PPEs, Writebol says she wore gloves and a disposable apron; she was separated from workers exiting the treatment unit by a line that she never crossed. Thinking back, she believes a co-worker who did the same job may have infected her. He became ill with what he thought was typhoid; he died from Ebola. "I never remember touching him," she says—but it's possible she picked up a sprayer he had used.

When Writebol first developed a fever on 22 July, she thought she had malaria, which a test confirmed. Her husband, David, cared for her while they continued sharing a bedroom. But she could not shake the fever, and 4 days later, a doctor gave her an Ebola test "to relieve everyone." After the results came

back positive, she was isolated, and David began speaking to her through a window near her bed. He did not develop Ebola.

An Ebola outbreak in Uganda in 2000, which Bausch helped bring under control, yielded some clues about the risks that infected people pose. Bausch and co-workers studied samples from 26 patients. In acute cases, the virus turned up most often in saliva but was also present in stool, tears, nasal blood, and breast milk. Although their sample sizes were small, the team did not find it in sweat or urine, and Bausch says he doesn't think people well enough to walk around the streets secrete the virus in those fluids—which means something like a handshake probably presents little risk. In one recovered patient, the virus turned up in semen 40 days after the onset of his disease.

Bausch says other studies have clearly shown that sicker people have higher viral levels. Corpses have the highest levels of all, and the virus “will seep into other tissues to saliva or sweat,” he says, putting family members and burial teams at risk. Environmental surfaces—unless they’re “grossly contaminated with blood”—are unlikely sources of transmission, he says. “It’s not jumping off the walls or hanging around when there’s not infectious bodily fluid there.”

Rigorous training can bring down the risk of infection. Doctors Without Borders (MSF), which literally has written the book on operating an Ebola treatment unit, has so far had only one worker contract the disease despite taking care of the majority of patients in this epidemic. Last week, the U.S. Centers for Disease Control and Prevention (CDC) held the first of what will be many 3-day training courses in Anniston, Alabama. It took place at an old Army base where working conditions resemble those in the affected countries, including a hot climate with no air conditioning. CDC’s Michael Jung, who is leading the program, says nearly every trainee had breaches of protocol, such as skin showing. Bausch attended the session, along with MSF staffers, to share some firsthand stories.

Adequately staffing Ebola treatment units also helps reduce the risk. At the training, Bausch recounted his work as one of two doctors in a 55-bed treatment unit. “You go into that ward and there are probably five or 10 patients who have fallen out of bed or are in delirium and have crawled out, there’s blood and vomit and diarrhea everywhere,” he says. “And there’s no one with a sprayer behind you cleaning it up.”

Still, when everything is done right, working in an Ebola treatment unit need not be a life-threatening endeavor, Bausch stresses. “Otherwise, I wouldn’t do it myself and it wouldn’t be ethical for me to counsel other people to do it.” ■



Tsunami waves ravaged Taro, Iwate, Japan; was a massive undersea landslide to blame?

GEOSCIENCE

Double-whammy tsunami?

Japan's 2011 quake may have had a hidden accomplice

By Roland Pease

On 11 March 2011, a magnitude-9 earthquake jolted northern Japan and sent a devastating tsunami sweeping down the coast, overwhelming seawalls with a surge more than 10 meters high. Along one 100-kilometer stretch of mountainous coastline called Sanriku, however, the incoming waves reached 40 meters. Those monstrous waves claimed about a quarter of the tsunami's 18,000 victims, yet experts have struggled to explain them.

Now, an international team of researchers says its computer models suggest a previously unsuspected answer: An underwater landslide the size of Paris combined with waves from the quake to deal the coast an extra-deadly blow. But others say they'll need stronger evidence to convince them.

Geoscientists have long known that undersea landslides can trigger tsunamis. But most saw no evidence that one had accompanied the 2011 quake. Instead, seismologist Kenji Satake of the University of Tokyo's Earthquake Research Institute proposed that an undetected second earthquake, involving a thin sliver of crust, had struck north of the main submarine thrust.

But Stephan Grilli, an oceanographer at the University of Rhode Island, Narragansett Bay, says fault movements don't jolt the sea surface in the right way to focus a band of waves as narrowly as at Sanriku. In the new study, he and colleagues worked back from details of the water motion recorded by gauges along the Japanese shore on the day of the earthquake to infer the ocean floor disturbance responsible. They conclude that a slab of sediment measuring 20 by 40 kilometers and up to 2 kilometers thick slid

about 300 meters down the steep slope of the Japan Trench, “acting like a piston.”

Grilli estimates that the slump must have happened near the northern end of the 2011 rupture, 170 kilometers from the Japan shore, and under 4.5 kilometers of water. A co-author, marine geologist David Tappin of the British Geological Survey, compared Japanese seafloor maps from before and after the earthquake and saw signs of just the right kind of slump in the target area. The team's paper is in press at *Marine Geology*.

The authors make a good case but are far from proving it, says Costas Synolakis, a tsunami expert at the University of Southern California in Los Angeles. Synolakis collaborated with Tappin and Grilli on previous studies that showed a similar slump caused a deadly 1998 tsunami off Papua New Guinea. This time, however, he worries the researchers are fixated on details of the tsunami modeling at the expense of the big picture. “Anyone who thinks you can model the behavior of a tsunami to better than a factor of 2 is crazy!” he says. A detailed survey of the sea floor would settle the case, he says. Satake, however, maintains that his two-quake explanation is adequate and that the existing seafloor mapping reveals nothing.

If a submarine landslide was responsible for the Sanriku surge, “then it's a game-changer,” says team member Robert Geller, a seismologist at the University of Tokyo. Geller has long criticized as unscientific the Japanese earthquake-forecasting program and hazard maps based on it. If towering tsunamis can also be produced by collapses along the Japan Trench, he says, there's little hope of anticipating the next one. ■

Roland Pease is a writer based in Swindon, U.K.

PHOTO: HITOSHI KATANODA/POLARIS/NEWS.COM

Intruder from the Oort cloud will graze Mars

Five spacecraft will have front-row seat for the encounter with comet Siding Spring

By Eric Hand

On 19 October, the comet Siding Spring will brush past Mars in a near miss. It will come as close to the planet as a third of the distance between Earth and the moon—in full view of a flotilla of Mars-orbiting spacecraft. After cowering from high-speed dust in the comet's tail, the spacecraft will get a once-in-human-history look at the comet itself, and at what happens to the martian atmosphere as the comet's diffuse, gassy envelope collides with it.

"If this had happened 50 years ago when I was a kid, we wouldn't have any outposts at Mars," says Carey Lisse, an astrophysicist at the Johns Hopkins University Applied Physics Laboratory in Laurel, Maryland. "We shouldn't be seeing this." Five orbiting spacecraft—two of which arrived just last month—will be in the rare position to observe the pristine comet. Since its formation some 4.5 billion years ago, Siding Spring has never before entered the inner solar system and been altered by the heat of the sun.

Discovered by Australian astronomers in January 2013, Siding Spring (technically known as C/2013 A1) does not orbit the sun in the plane of the solar system, as all other comets visited by spacecraft have done. Those comets have orbits with periods of tens or hundreds of years, and they hail from the Kuiper belt, a flat, ring-shaped region beyond Neptune. Siding Spring, by contrast, has had a looping orbit that takes several million years to complete, an indication that it comes from the Oort cloud—a much more distant, spherical shell that planetary scientists think harbors trillions of comets. Astronomers suspect that this is Siding Spring's first foray into the inner solar system, because of both its orbit and signs early on that the sun had turned on jets of carbon monoxide and carbon dioxide gas. In a repeat visitor, stores of these easily volatilized ices would have been exhausted already.

The gas and dust spewing from the comet worried Mars mission managers at first. Traveling at 56 kilometers per second, even a millimeter-sized dust particle

could scupper an orbiter. NASA organized several teams to model the behavior of the comet's dust tail. They found that the peak risk would come 100 minutes after the comet's closest approach and would last about a half-hour. Tony Farnham, an astronomer at the University of Maryland, College Park, who led one of the modeling efforts, says the worst-case hazard is a flux of one millimeter-sized particle through an area of 10 square kilometers.

Nonetheless, many of the orbiters are taking precautions. Three NASA spacecraft—Mars Odyssey, Mars Reconnaissance Orbiter (MRO), and MAVEN (Mars Atmosphere and

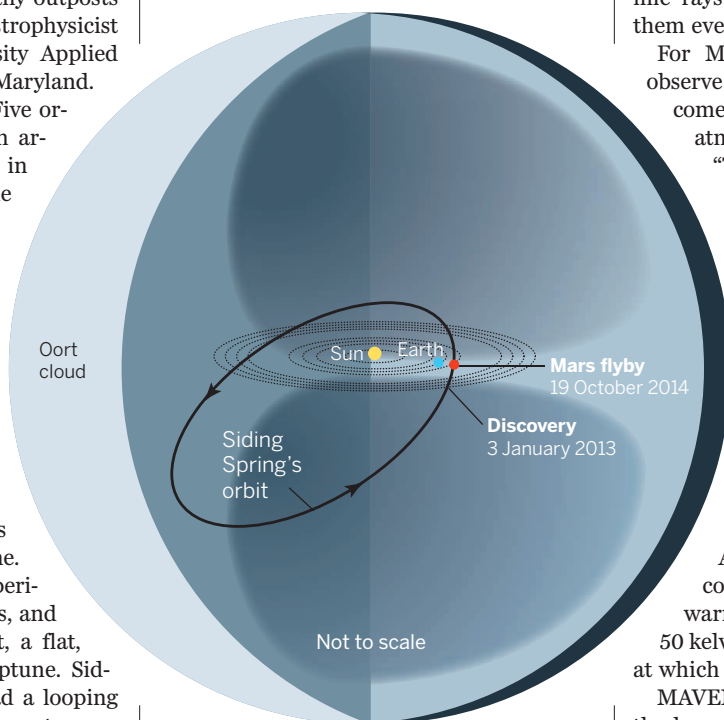
able to see the comet's nucleus, which unless viewed up close is veiled by its gassy atmosphere, or coma. Resolving an Oort cloud comet nucleus would be a first—and there are reasons to think such comets could be larger than their Kuiper belt counterparts. Also highly sought is a measurement of the nucleus's albedo, or reflectivity. Covered in a crust of dark, organic-rich dust, most comets are coal black. But Oort cloud comets, with their stores of primitive ices that have not yet been sublimated away by repeat encounters with the sun, could be slightly brighter. Alternatively, Lisse says, 4.5 billion years of being "cooked" by cosmic rays in the solar exurbs might leave them even darker than normal.

For MAVEN, a spacecraft designed to observe Mars's thin atmosphere, the comet—also a body with a wispy atmosphere—will be an ideal target. "The spacecraft doesn't care if it's a planet or a comet," says Karl Battams, an astrophysicist at the U.S. Naval Research Laboratory in Washington, D.C.

Even better will be when the two atmospheres collide. Gas molecules in the large coma—mostly water—will slam into Mars at energies of 300 electron volts, says Roger Yelle, a MAVEN team member and a planetary scientist at the University of Arizona in Tucson. Yelle says the collision will trigger aurorae and warm the martian atmosphere by 50 kelvin, temporarily driving up the rate at which it erodes to space.

MAVEN is specifically designed to study the loss of the martian atmosphere, which was much thicker billions of years ago. Team members had always hoped to watch how it responds to special solar events. "We were hoping for some big solar flares," Yelle says. "Instead of a flare, we get a comet." The two types of events should perturb the atmosphere in remarkably similar ways, he says, and will help illuminate the processes at work during the erosion of the atmosphere.

Just a few days later, the comet will have whipped up out of the plane of the solar system and receded from view of the spacecraft, not to return for another million years or so. ■



Siding Spring's near-miss of Mars will be a rare chance to see an Oort cloud comet up close.

Volatile EvolutionN)—have adjusted their orbits to swoop behind the planet during the worst 40 minutes of the encounter, as has the European Space Agency's Mars Express. MAVEN, which arrived on 21 September, will take the extra step of turning off some of its high-voltage instruments. Plans for India's Mars Orbiter, which arrived on 24 September, are still being worked out.

Both before and after the spacecraft take shelter, their eyes will be on the comet. Researchers' dearest hope is that MRO will be



Before falling into disrepair after independence, the extensive rail network in the Democratic Republic of the Congo likely spread HIV.

VIROLOGY

Early AIDS virus may have ridden Africa's rails

Genetic study reveals fresh details on HIV's emergence

By Jon Cohen

Sometime around the early 1900s, the virus that sparked the AIDS epidemic likely spread from a chimpanzee to a human in southeastern Cameroon. In roughly 1920, someone infected with it traveled down the Sangha River and its tributaries from Cameroon to Léopoldville, today known as Kinshasa, the capital of the Democratic Republic of the Congo (DRC). A report on page 56 spells out what happened next: how and when HIV left Kinshasa and spread through Central Africa in the first stage of an epidemic that less than a century later has infected nearly 75 million people worldwide.

The work, which highlights how Africa's rail network probably helped spread the virus, also proposes why one particular version of HIV far outpaced others. "There were lots of different factors," says study leader Oliver Pybus of the University of Oxford in the United Kingdom, who specializes in the evolution of infectious diseases. "Basically this one was at the right time and the right place—and it hit the jackpot."

Previous studies have looked at differences between simian and human immunodeficiency viruses to piece together

HIV's very early history, including its origin in Cameroon. Basically, HIV's initial emergence has become clear by comparing chimpanzee counterparts with traces of the AIDS virus uncovered in archived tissue or blood samples from as early as 1959 in Léopoldville.

While some researchers continue to search for even older human tissue samples containing HIV, the new analysis relies exclusively on information already deposited in the HIV Sequence Database maintained by the Los Alamos National Laboratory in New Mexico. The initial goal of the study was to address a little-appreciated curiosity about the AIDS virus. Researchers have documented 13 different cases in which a simian immunodeficiency virus has jumped from monkeys, chimpanzees, or gorillas into humans. But only the virus known as HIV-1 group M (for "major") traveled far and wide and created an epidemic. What gave it the advantage?

Looking for an answer, Pybus and colleagues examined the genetics of HIV samples from various African locations over the past half-century. They started with 348 samples of group M viruses from the former Belgian Congo, renamed Zaire and then DRC, and 466 more from neighboring

countries. Then they used the relatedness of the sequences to create family trees, or phylogenies. Next they applied a "molecular clock"—the known rate at which retroviruses such as HIV mutate—to date the origin of each tree and its branches.

The new analysis confirms that HIV sequences have a common ancestor dating back to about 1920, as the earlier studies suggested. "It's really nice that a new group of investigators has worked on this using the most cutting-edge molecular phylogeographic methods and come up with the same conclusions," says Beatrice Hahn, a virologist at the University of Pennsylvania who has done pioneering studies of HIV's origins.

After it reached Kinshasa, the new study suggests that trains played a major role in helping the virus, reaching Lubumbashi (then Elisabethville) around 1937 and Mbuji-Mayi (then Bakwanga) 2 years later. (This level of precision comes from comparing the many HIV sequences from both of those locations in the Los Alamos database with other HIV sequences from different times and places.) Although earlier analyses had assumed HIV expanded its range mainly with the help of riverboats, Pybus and co-workers note that Mbuji-Mayi and Lubumbashi were major mining centers, connected to other parts of the country by rail lines that carried hundreds of thousands of passengers per year until they fell into disrepair after independence.

Jacques Pépin, a co-author of the paper and an epidemiologist at the University of Sherbrooke in Canada, says the apparent role of the railway surprised him. "When I lived in Zaire in the early 1980s, the railway system had already largely collapsed,

nowhere to be seen,” says Pépin, who in 2011 published a book, *The Origins of AIDS*. Pépin and colleagues think Mbuji-Mayi and Lubumbashi may have historical collections of tissue and blood samples that could add more detail about group M’s early years. They suspect, for example, that Mbuji-Mayi is the birthplace of one variant of group M, known as subtype C. That subtype spread widely on the continent because of migrant labor and today accounts for roughly half of the infections in sub-Saharan Africa.

Another variant, subtype B, which accounts for most HIV infections in the United States and Europe, surfaced in Kinshasa around 1944, the new study shows. It also bolsters earlier assertions that the subtype infected Haitian professionals who came to DRC in the ’60s and then carried it home in about 1964.

From 1920 to 1960, group M and an outlier group of HIV variants designated “O,” which is largely restricted to Cameroon, spread at similar rates, the paper finds, but then group M’s growth rate nearly tripled. The researchers contend that group M’s sudden success outpaced the rate of population growth in Kinshasa, so that cannot explain why it shot ahead of group O. They suggest instead that commercial sex workers in Kinshasa had more clients than those elsewhere or that public health campaigns there—maybe directed at sexually transmitted diseases—did not properly sterilize injection equipment. Neither idea is based on hard data, but the researchers do present intriguing evidence for the injection scenario: A study led by Pybus last year reported that men in DRC older than 50 years were far more likely to have been infected with hepatitis C virus, which readily spreads through contaminated needles.

Michael Worobey, an evolutionary biologist at the University of Arizona in Tucson who has published extensively on HIV’s origins, calls the new work “technically brilliant” but stresses that there are still more mysteries to solve. “I don’t think this paper nails down the different experiences of group O and group M viruses,” Worobey says. He’s particularly doubtful that unsterilized injection equipment played a key role in the initial spread, noting that the public health campaigns in colonial Congo date back to long before group M took off.

Still, Worobey says it’s “miraculous” that studies continue to clarify the origin of the AIDS epidemic. “Who would have thought that if you just lined up all the ACTGs of DNA sequences from this virus found in people over different time points you could tell what was happening in colonial Africa and how this thing unfolded?” he asks. ■

QUANTUM MECHANICS

Breakthrough lost in coin toss?

A controversial quantum measurement that seems to bend the rules may not be very quantum after all

By Adrian Cho

For 26 years, physicists have argued over an unorthodox quantum measurement technique that seems to circumvent one of the central tenets of quantum mechanics. “Anomalous weak values” bend the rule that you can’t measure a quantum particle without disturbing it, devotees say, and provide deep insights into quantum reality (*Science*, 5 August 2011, p. 690). Skeptics counter that the whole enterprise is just standard quantum mechanics misinterpreted. Now, with the toss of a coin,

who developed the critique of anomalous weak values with Joshua Combes of the Perimeter Institute for Theoretical Physics in Waterloo, Canada. But the inventors of the technique dismiss Ferrie and Combes’s argument, published on 18 September in *Physical Review Letters*.

One illustration of the rule that you can’t measure a quantum particle without disturbing it is a silver atom, which acts like a magnet that can point up, down, or, thanks to the weirdness of quantum mechanics, both ways at once. Try to measure whether the atom is pointing up or down, and that

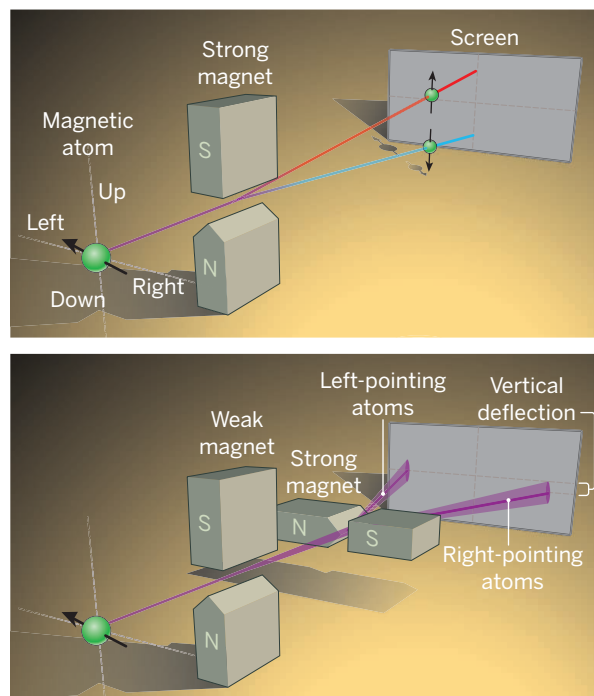
both-ways state “collapses” one way or the other. For example, imagine starting with atoms in a state that is close to “up plus down”—which is the same as pointing to the left—and firing them through a vertical magnetic field (see figure, left). The field splits the beam, and the numbers of atoms in the upper and lower beams reflect the proportions of up and down in the original state—your measurement. But the atoms in the upper beam point only up and those in the lower beam point only down because their states have collapsed.

However, in 1988, Yakir Aharonov of Chapman University in Orange, California, and Lev Vaidman of Tel Aviv University in Israel figured out how to glean such information without collapsing the state into up or down. First, weaken the vertical magnet so it merely widens the beam—a nonmeasurement. Then add a strong horizontal magnet to split

the beam left to right. Because the atoms started off pointing toward the left, only a few of them exit to the right. But in that “postselected” right beam, the lingering effect of the weak vertical magnet makes the up and down parts of the quantum state interfere like overlapping waves to deflect the beam vertically. That deflection serves as

Measurement with a gentle touch

A normal measurement (top) splits a beam of atoms while changing their quantum state. A weak measurement (bottom) exerts a milder influence.



two theorists argue that anomalous weak values aren’t inherently quantum mechanical and therefore offer no novel insight into the quantum realm.

“There is a classical scenario in which you can get the exact same result as in the quantum scenario,” says Christopher Ferrie of the University of New Mexico, Albuquerque,

a “meter” to reveal the amounts of up and down in the original state. And, as has been demonstrated in the lab, the deflection can be oddly large, as if the atom has 100 times its usual magnetism. So the theorists dubbed it an anomalous weak value.

The amplification effect could be used to make, say, precision frequency measurements. And by avoiding disturbance, the technique may allow researchers to sidestep some of the paradoxes of quantum theory. For example, in a classic experiment, photons pass through a plate with two slits. Left unperturbed, they act as a wave, which passes through both slits at once and then interferes with itself to create a striped pattern on a distant screen. But that pattern disappears if you try to trace each photon's path through one slit or the other. That's because the photon can act like a wave or a particle, but not both at the same time. However, in 2011, experimenters used a weak values scheme to trace the average trajectories, summed over many photons, in the experiment without destroying the pattern.

Others have used weak values schemes to try to make sense of even more paradoxical examples of wave-particle duality. The key is always to find some variable that links only weakly and uncertainly to a particle path—for a photon, perhaps its polarization—and to choose just the right postselected state to filter out and amplify that effect. Everybody agrees on how to set up the experiments. The controversy arises when Aharonov and others contend that the weak values are not just unusual experimental readings, but also elements of quantum reality. Indeed, Aharonov goes so far as to argue that the schemes show that quantum states run both forward and backward in time.

Critics dispute these claims. The amplification effect is an artifact of postselection, they say. The final step of the process does collapse the quantum state, albeit into the particular postselected state. And weak values can be determined only retrospectively after many trials, so all the quantum states simply ran forward in time until the experiment ended.

Now, Ferrie and Combes say that the weak effects aren't even a quantum phenomenon. They propose a thought experiment in which two people, Alice and Bob, produce an anomalous weak value classically. Instead of fiddling with atoms and their both-ways states, Alice repeatedly hands Bob a simple coin, heads up. Bob measures its state, but only weakly and uncertainly—perhaps he can only feel it with his thumb. Crucially, Bob's measurement also disturbs the coin and flips it with different probabilities depending on whether he detects heads or tails. He announces his result and hands

the coin to Alice, who tallies the numbers of times Bob says “heads” or “tails.” The difference in the sums serves as the meter weakly measuring the state of the coins.

The weaker Bob's measurement, the more often he will say “tails” and the smaller the difference in the tallies of heads and tails will be. However, if Alice postselects events in which Bob has flipped the coin to tails, then the difference can be sizable, even as the measurement becomes

“This is a perfect example of how you can have beautiful mathematics and lose the physical intuition.”

Yakir Aharonov, Chapman University

very weak. That constitutes an anomalous weak value, Ferrie and Combes argue. The key is that Bob's measurement has to flip the coin with different probabilities depending on whether he detects heads or tails. That's why Alice will see the effect only if she does the postselection. Still, the anomalous weak value emerges in a classical way, which means weak values cannot provide novel insight into the quantum realm, Ferrie and Combes say.

Aharonov disagrees. “This is a perfect example of how you can have beautiful mathematics and lose the physical intuition,” he said at a 19 August seminar at the Perimeter Institute at which Ferrie presented the work. Anomalous weak values result from interference of quantum states, Aharonov insists. And he contends that in his original scenario the atom really has 100 times its ordinary magnetism in the time between the initial and final states. “There's no classical wiggling that will produce a magnetic moment that's 100 times larger,” he said at the seminar.

However, Anthony Leggett, a theorist at the University of Illinois, Urbana-Champaign, says Ferrie and Combes have made a good case that anomalous weak values are an artifact of a disturbance and postselection. But he cautions that Ferrie and Combs have not proved that all the experiments done with weak values have classical explanations. “One ought to look at the individual case in detail,” he says.

Aephraim Steinberg, the experimentalist at the University of Toronto in Canada who applied weak values to the two-slit experiment, says he's “still mulling it over.” He notes that the debate has never been over whether the experiments work as predicted: “We all agree on the experiments, we just don't agree on what they mean.” ■

FEATURES



The littlest patient

Cutting-edge mouse models fuel hope for understanding and treating cancer

By Jennifer Couzin-Frankel, in New York City

The worst day of Kenneth Olive's career began unremarkably. He woke up in his two-bedroom apartment in Harlem and tag-teamed breakfast for his 1-year-old son as he and his wife raced to get ready for work. At the 116th Street subway stop nearby, Olive hopped on a C train uptown to 168th Street.

His lab is about a block away, in the cancer center at the heart of Columbia University's medical complex. There, the young assistant professor was banking his career on a mouse—one he hoped would pinpoint new drugs for pancreatic cancer, among the deadliest diagnoses in oncology.

Sitting on the train, Olive opened an e-mail message he had downloaded earlier but hadn't yet read. It was from an executive at a biotech company with which he was working closely. What he read sucked the air right out of him. The company, Infinity Pharmaceuticals Inc., had been running a clinical trial in 122 people with advanced pancreatic cancer; about half were getting a new drug thanks to impressive results in Olive's genetically engineered mice.

Days earlier, data monitors had noticed a striking disparity between patients who were on the experimental treatment and those who were not—but because the trial was blinded, they did not know which group was which. Now, the blinds had been lifted. To everyone's horror, the patients dying more quickly were on the new drug. It was the first big test for Olive's mice—and the animals had, by all appearances, failed spectacularly.

Kenneth Olive holds one of his mice engineered to develop pancreatic cancer. He tests drugs on the animals in hopes of identifying the best ones for cancer patients.

"I remember being numb," Olive says. It was January 2012, 2 years since he had joined Columbia's faculty. He was 35 years old. "We had built our entire laboratory based on this mouse," he says. Intended to mimic human cancer with unusual precision, the animals were even being monitored and treated in a "mouse hospital" custom-built just for them. Rushing into work, Olive called a lab meeting for 10:30. After his group gathered, he broke the news. "It was a very quiet and heavy room. I told them that we were allowed to be mopey for 3 hours, and then we were going to have a meeting that afternoon to brainstorm" what went wrong.

Olive is trying to shift a dismal statistic that plagues his field: About 90% of cancer drugs that enter clinical trials based on

"I told them that we were allowed to be mopey for 3 hours, and then we were going to have a meeting that afternoon to brainstorm."

Kenneth Olive, Columbia University

upbeat mouse data fail. For some tumors, the need for new therapies is especially acute. In pancreatic cancer, the 5-year survival rate is about 6%. "These patients," Olive says, "don't have any options."

To change that, he and a growing number of other cancer researchers are trying to build a better mouse. Their approaches are a radical break from the past: taking human cancer cells grown on plastic over many years and injecting them into an animal. Dozens if not hundreds of drugs have subdued cancer in these mice. A handful have done the same for people.

Olive believes he can do better with a genetically engineered mouse. At conception,

he endows the animal with the same gene mutations that show up in most human pancreatic cancer cells; the mutations become active during early development, in cells destined to form the pancreas. Just like certain people, these animals spontaneously develop pancreatic tumors.

Changing the paradigm requires not just a superior rodent. It also takes rethinking how and how thoroughly the animals are studied. To wring as much information as possible from his mice, Olive schedules every one of them for ultrasounds twice a week to check for tumors and monitor existing disease. The "treatment room" includes boxes of surgical gloves, sterile drapes, a surgical microscope, and a venting hood to protect researchers from inhaling the anesthetic they use on the animals.

But that day in 2012 underscored just how difficult it is to mirror human cancer in a wriggling 30-gram ball of fur, where tumor size is measured in millimeters and lifespan in days. Every couple of weeks Olive hears from a scientist or company eager to test a favorite therapy. "Can you just throw this into a few mice for me?" they ask. That isn't quite how it works, Olive says. The risks and the challenges sometimes feel overwhelming. But the payoff, he believes, will make it all worthwhile—if his mice pan out.

OLIVE STEPPED INTO THE WORLD of pancreatic cancer by chance. In 2005, fresh out of graduate school, he was recruited to join the lab of David Tuveson, an oncologist and cancer biologist then at the University of Pennsylvania. Tuveson had just published a paper describing an uncommonly accurate mouse model for pancreatic cancer.

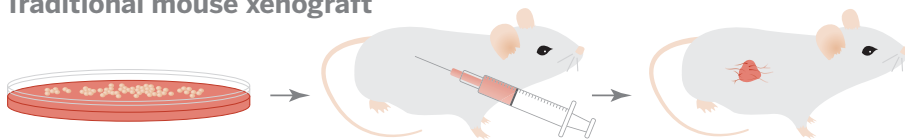
To create it, Tuveson mutated two of the mouse's genes. One, *Kras*, shows up in about 95% of human pancreatic tumor samples. The second, *p53*, appears in 70%. The model isn't an exact replica of humans. Unlike people, the animals have these mutations throughout their pancreas, not just in their tumor cells, and throughout their life rather than just when cancer makes its appearance.

Yet the mice captivated Tuveson and Olive. The disease's choreography closely matched its dance in people. It metastasized to the same sites—the liver, the lymph nodes, the lungs. The animals developed many of the same complications as people, including fluid collections in the abdomen and a muscle-wasting syndrome. When Tuveson slipped a slide from a mouse's tumor into a stack of slides from people, a pathologist "couldn't tell the difference," Olive says. But would a mouse whose cancer generally

Three model mice

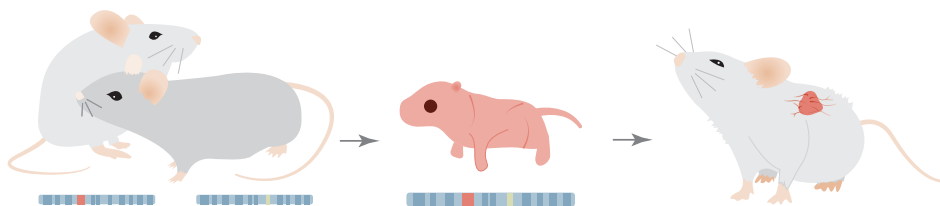
Traditional mouse xenografts have been used for years as cancer models, but many researchers say they can do better. They are experimenting with mice genetically engineered to develop cancer and ones that carry a patient's specific tumor.

Traditional mouse xenograft



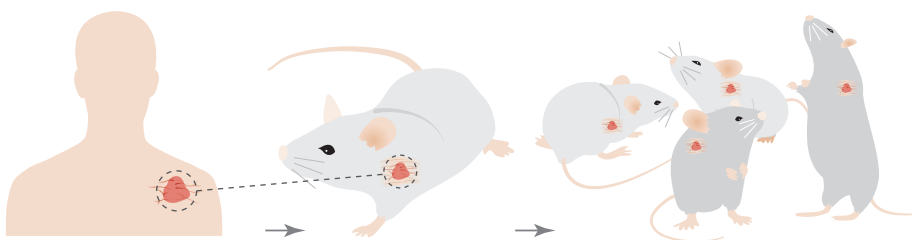
Cells from a human tumor are grown in the lab and maintained for many years. They are then injected into a mouse or in some cases inserted surgically into certain organs.

Genetically engineered mice



In one version of this model, two types of mice are engineered: one with the mutated genes that are inactive, and one with an "activator." When the animals are bred, the offspring carry active mutations that lead to a specific cancer.

PDX mice



Tumor and surrounding tissue from a patient are implanted into a mouse. After they engraft, tumor samples from that mouse are removed and implanted into others, creating a cohort with the patient's tumor.

resembled a human's and behaved like it also respond similarly to therapy? "You have to go into it without any assumptions," says James Doroshow, who directs the division for cancer treatment and diagnosis at the National Cancer Institute (NCI) in Bethesda, Maryland. NCI is working on both genetically engineered models and another called patient-derived xenograft (PDX) models, in which tumor and some healthy tissue are removed from a patient and engrafted into a mouse (see p. 28).

Olive, just beginning his postdoc, was eager to plunge ahead with the new model. "I said, 'Great, I'm here, I want to put drugs' in these mice. 'Let's go.'"

Hang on, Tuveson shot back. How do you know which animals have cancer?

Olive paused. As he now explains, the mice "don't let you know that they're sick until they're very sick—and then you only

have a few days" until they die. Olive wasn't much interested in imaging, but he didn't have a choice. He began running around the university, "begging and borrowing" time on different machines—some built for mice, some for people—to visualize what, if anything, was growing deep inside the animals' bellies.

In a stroke of luck, he happened upon an ultrasound machine designed for rodents in the cardiology department. He asked if he could bring one of his mice along and discovered the machine was just right. It was small, easy to use, and, by the standards of such equipment, relatively cheap—about "\$350,000 instead of \$3.5 million," says Olive wryly. Tuveson, a young faculty member, pooled money from his startup package and grants to buy one for the lab.

Now, Olive was ready to put the mice through their paces. The obvious starting

point was the chemotherapy drug gemcitabine, which until the mid-2000s was the only therapy approved to treat pancreatic cancer. Like so many before it, it had performed impressively in early mouse trials, but only about 5% to 10% of patients benefit from it.

Other researchers developing cutting-edge mouse models have taken a similar tack: testing whether their models mimic known human drug responses. At the University of Turin in Italy, molecular oncologist Livio Trusolino gathered colon cancer tissue from patients whose disease had spread to their liver and engrafted this tissue into animals without a functioning immune system to create PDX mice. Then, he gave the animals an antibody that's often used in patients. The proportion of mice whose tumors shrank or stopped growing "were pretty much the same as the ones in the clinic," he says. In Boston, researchers tested a genetically engineered mouse model for lung cancer in parallel with an AstraZeneca clinical trial of a targeted therapy. The mice, which had the same genetically driven disease subset as the patients, received the same treatment. "The moment that was striking for me was when the human data were announced, and our data matched exactly," says thoracic oncologist Kwok-Kin Wong at the Dana-Farber Cancer Institute, who led the work.

Olive hoped for a match, too. Most scientists want to see their drug cure mice. He was praying his would fail.

It did. Tumors shrank in just two of 17 animals. Flush with success, Olive proceeded to his next drug: Infinity's.

"THESE ARE OUR OUTPATIENTS," Stephen Sastra says with a sweep of his hand. "These are our inpatients. And this is our maternity ward." Sastra is a research scientist originally from Melbourne, Australia, who has spent 15 years operating on small animals. The mouse hospital, with its racks of cages and musty smell, is his domain.

Clad in protective booties, gloves, a yellow gown, and a hair covering, Sastra keeps a close eye on more than 750 animals. Some (the "outpatients") are healthy but, thanks to their mutations, at high risk of cancer; others are sick; and others are nursing their newborns, the next wave of study enrollees. Real estate in New York is at a premium even for mice, so the Olive lab owns another 207 animals in a facility in Massachusetts.

Some mice with cancer have a biopsy at diagnosis, so that the tumor can later be compared with its treated version—in humans and mice, cancer cells often change after treatment. That can help the researchers decipher why a drug succeeds,

or fails. After gemcitabine flopped in their mice, as they'd hoped it would, Olive and Tuveson found that the stroma—the healthy cells surrounding the tumor—shield the cancer from chemotherapy. In a 2009 paper in *Science*, they speculated that this might help explain why so many drugs don't help pancreatic cancer patients.

Other scientists are using revamped mouse models to explore the diversity of cancer in people. “Personalized medicine and targeted therapies in fact stand on exceptions,” Trusolino says. He is particularly interested in patients who are resistant to drugs, especially those whose resistance can't be explained by known genetic mechanisms. When he gave his PDX mice an antibody commonly used in metastatic colon cancer, a subset didn't respond, just as happens in people. Trusolino found that about 20% of these nonresponders had alterations in a gene called *HER2*, which is a drug target in breast cancer. He experimented with giving them two breast cancer drugs. The tumors receded. A clinical trial, called HERACLES, is now enrolling colon cancer patients with the same alterations to test the same drugs. Results aren't out yet, but Trusolino says tumors are shrinking or stable in many patients. “It is the first time in my life that I see results from my lab translated into a clinical benefit,” he says.

Knowledge like this can inspire streamlined clinical trials. It can also clarify how to use drugs already on the market. “Sometimes we're catching up,” says William Sellers, vice president and global head of oncology at the Novartis Institutes for BioMedical Research in Cambridge, Massachusetts. “We are going back and doing this to get more data on drugs we have.”

Most believe that these new mice adhere far more closely to human biology than did their predecessors. But they are not without pitfalls. In PDX mice, the healthy human tissue, or stroma, that's transplanted is replaced over time by a mouse version, and it's not clear if that alters drug responses. The animals lack a functioning immune system—otherwise, the transplanted human tumor won't engraft. But that limits which drugs can be tested, ruling out immunotherapies, and it may also affect how closely the mice reflect human biology. In the genetically engineered mouse, the tumors may lack the genetic diversity seen in people, and the animals often have lesions scattered throughout their organ. “I think we've got to stop looking at models as faithfully recapitulating disease,” says Carol Bult, scientific director of the PDX program at the Jackson Laboratory in Bar Harbor, Maine. “You pick a model to address the question you're asking.”

What's more, the animals aren't for everyone. Each of Olive's mice comes with a \$1380 price tag from breeding until death. His mouse hospital costs many hundreds of thousands of dollars a year to run. There is also a high cost in time: for PDX mice to engraft the tissue, and for genetically engineered mice to develop cancer. Olive's animals live 5 1/2 months on average, which, he says, “is a long time sitting on a shelf.”

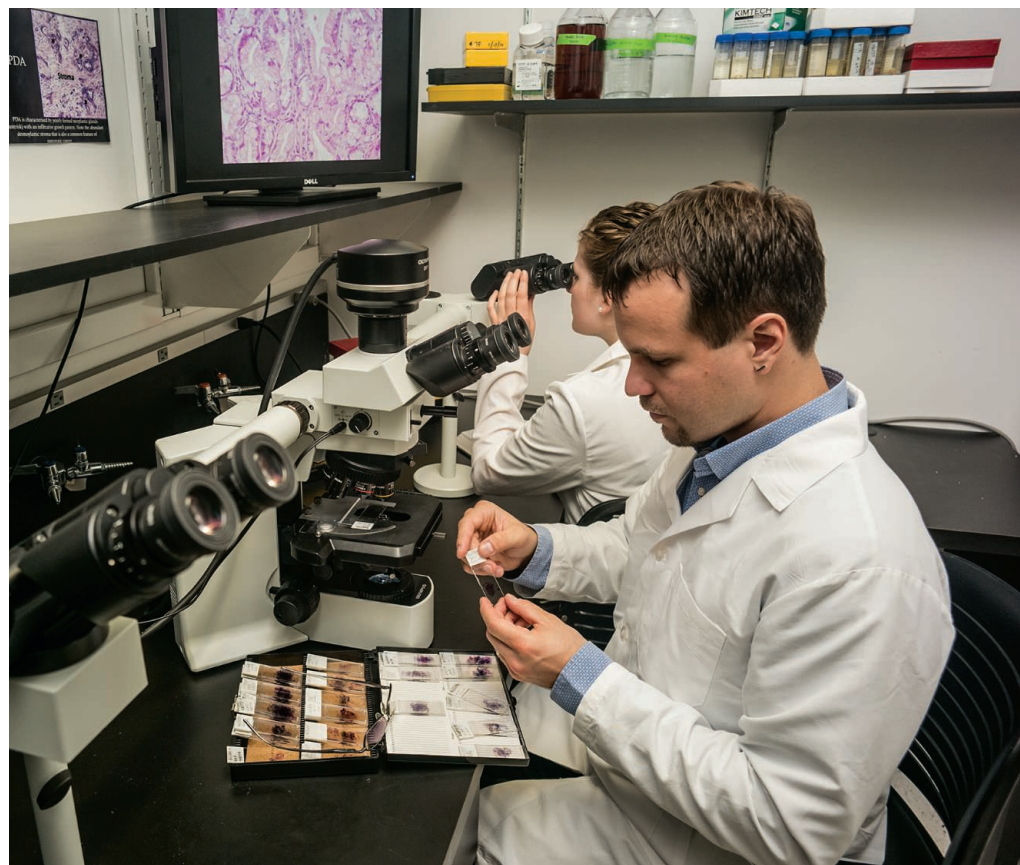
FAILURE PROPELS OLIVE FORWARD.

After the Infinity news broke, his dejected group generated 21 hypotheses, then whittled them down with the help of patient

as the others. The animals' tumors, he also discovered, were poorly differentiated, a type that grows and spreads especially quickly.

The therapy had been designed to disrupt the stroma in the hope that doing so would make it easier to treat with chemotherapy. In the short term, that worked. In the long run, it destabilized the cancer and changed its pathology.

Olive could breathe again. His mice, and his career, were safe for now. But the episode exposed how easily everyone had misread the original experiment. Although the animals had lived an extra 2 weeks, any tumor regression had been fleeting. The



Probing cancer biology, Kenneth Olive and graduate student Jaime Eberle examine pancreatic tumor samples from their genetically engineered mice.

data shared by the company. Ultimately, one theory rose to the top. The animals were treated for a few weeks. The patients were treated for months. What if there was an acute response to the drug that was positive, Olive wondered, and a chronic one that was not? They began a new experiment, enrolling mice with precancerous lesions and treating them longer.

“One hundred percent were dead by 4 months,” Olive says. “It was a dramatic difference” from the study that had swayed Infinity, in which the mice getting the drug lived twice as long after the study began

experience “changed my outlook on what constitutes a response” in a mouse, Olive says. He now wants to see impressive tumor shrinkage, not just slower growth.

At lunch one Wednesday in early September, Olive ran into an oncologist colleague at a sandwich shop across the street. The two chatted amiably. That doctor, Olive later shared, is caring right now for a 28-year-old with pancreatic cancer. It's “unheard of” for the disease to strike someone so young, he says. These are the stories that keep him focused on saving patients, one mouse at a time. ■



Robyn Stoller's husband, Alan, died as drugs were being tested in mice carrying his sarcoma. None worked well, but she takes comfort in knowing that "we tried everything."

Hope in a mouse

Selling personalized mouse models to cancer patients, a firm draws thanks and reproach

By Jennifer Couzin-Frankel

In the end, it became a race against time. "Maybe 5 days before he actually died, 4 days, I remember saying to him, 'Alan, you've got to hang on, you've got to hang on, the drugs are in the mice, they're looking at them,'" says his wife, Robyn Stoller. The couple had three children in elementary school and lived in a suburb of Washington, D.C. Alan, a 47-year-old who worked in real estate finance, had a virulent form of sarcoma that had spread to his brain.

Forty miles away, scientists at a company in Baltimore, Maryland, were pushing the limits of biology. They had implanted samples of Alan's tumor into mice without a

functioning immune system. Nine cohorts of animals received one of nine different drug combinations, a test of what might work best on Alan's tumor. But the process took months, time Alan didn't have. He died at home on 12 July 2010.

The journey to what Robyn calls "bleeding-edge technology" had begun almost 8 months earlier, on the couple's 13th wedding anniversary, when they drove to see oncologist David Sidransky at Johns Hopkins Hospital in Baltimore. Alan "was very sick," Sidransky remembers. "There was a lot of pressure not to treat him anymore."

The Stollers had sought out an alternative in Sidransky. He had recently formed a

company called Champions Oncology, its main lab a 15-minute walk away. Champions aims to personalize cancer care with mice that, Sidransky argues, are unusually predictive of how individual patients will respond to particular drugs. That's because each mouse *is* an individual patient—or rather, carries tumor harvested from one.

Champions is far from alone in building these mice, called patient-derived xenograft (PDX) models. The drug company Novartis has more than 1000 PDX models from as many patients, which it's applying to drug testing. The National Cancer Institute in Bethesda, Maryland, is developing a bank of PDX mice for rare cancers and patients enrolled in trials. The Jackson Laboratory in Bar Harbor, Maine, a major mouse supplier for academic and corporate labs, has more than 350. "There's a big push" for these mice, says Carol Bult, scientific director of the PDX program there, and many scientists are using them to examine tumor biology and drug resistance.

What most people aren't doing is selling the models to patients. The Stollers paid many thousands of dollars to Champions, which markets the animals as a treatment guide. The mice are called avatars, analogous to a computer game in which an avatar is a graphical representation of the person playing it. "There may be hope for your specific cancer," Champions assures patients on its website.

Many find this pledge troubling. The company is "selling a promise to patients before that promise has been judged to be true by really rigorous data," asserts George Demetri, an oncologist who treats sarcomas at the Dana-Farber Cancer Institute in Boston. Even though he advises his own patients against paying for avatar mice, Demetri is part of Champions' scientific advisory board. There, he says, he acts as a "gadfly" pushing for more and better studies to prove how predictive avatars are and whether any benefits are enough to justify the cost.

Right now, Champions charges \$2000 to create an avatar mouse, and about \$2500 for each drug test it runs—normally a recommended minimum of about three or four, with no upper limit. The company says it loses money on the personalized service; from April 2013 to April 2014, it brought in \$2.3 million but spent \$2.7 million, not including marketing and other indirect costs. Champions makes money off a separate business: testing

therapies on PDX mice for drug companies.

Sidransky acknowledges that with avatars, patients are paying for a strategy that's still experimental. But he sees no other way to offer it.

CHAMPIONS' STORY began with a single patient. About 8 years ago, Sidransky and another Hopkins oncologist, Manuel Hidalgo, were studying PDX mice. The tissue for the models came from Hopkins patients, but the mice were intended solely for research. That is, until a man with metastatic pancreatic cancer asked Hidalgo, "Can't we use the results?" for treatment? The oncologists sought permission from their institutional review board to transfer therapy from mouse to man. "It was all intuition," says Hidalgo, now an oncologist at the Spanish National Cancer Research Centre in Madrid. "Really, there were no data."

The patient lived almost 5 more years, unusually long for someone with his disease. "That wowed us," Sidransky says.

The pair began offering this boutique option to the small number of patients who had time to wait. Initially, it cost about \$50,000 per patient—funded by a clinical trial—and took about a year for mouse results to come in. "After 2 years of doing this, we decided to start a company," Sidransky says. In 2011, he and Hidalgo published outcomes on the 11 Hopkins patients they had treated. Fifteen of 17 treatments given (some people got more than one) "resulted in durable partial remissions," they wrote in the journal *Molecular Cancer Therapeutics*. "Overall, there was a remarkable correlation between drug activity in the model and clinical outcome."

Champions says it has replicated that study many times over. About 175 people have gone through the full process—avatars created, drugs tested on the mice, and information on those drugs offered to the oncologist. At the European Society for Medical Oncology meeting in Madrid this week, the company presented data on 102 drug regimens in 70 patients; 89% benefited from a given therapy if a mouse did, too, says Ronnie Morris, the company's president. The few correlations recorded by other researchers are roughly comparable to those claimed by Champions.

There are lingering questions, though, about what exactly "benefit" means—for instance, is it assessed by objective rules? "It is all about how one phrases the questions," Demetri notes, and the definition of "benefit" is "not a trivial concern." He is in discussions with Champions about designing clinical trials to address these issues. The first

of several Champions trials, focused on sarcoma, will launch this fall. All the studies will offer avatar mice at no cost and will track more systematically whether a mouse response matches a patient's. The company has no plans to perform a randomized trial, in which some patients receive the therapy their doctors recommend and others follow the mice.

"We can't be responsible for good and bad drugs," Morris explains. "We want to be an empiric test" of which drug a patient should take.

Avatar studies outside Champions are also picking up. Hidalgo is beginning a randomized trial comparing therapy directed by avatars against standard therapy in pancreatic cancer. At the Mayo Clinic in Rochester, Minnesota, oncologist Paul Haluska has created mice from about 400 ovarian cancer patients, with an eye toward successfully treating the relapses so many

cancer, you are misleading them because often, there is no time to play the game," says Pier Paolo Pandolfi, who is studying genetically engineered mouse models at Harvard University.

Although Champions has sped up the avatar-making process, it still takes about 4 months. Sidransky doubts it can get much faster. Many people die in that interim or become too sick to consider additional toxic therapy. This drop-off helps explain why more than 500 people have signed on with Champions, but only about 175 have been offered the option of mouse-inspired therapy.

Alan was in the two-thirds who don't reach the finish line. When he signed on with Champions, he was in such bad shape that he couldn't undergo surgery to gather fresh tumor tissue. Instead, the company convened an "expert panel" of sarcoma physicians and researchers who studied his initial biopsy, its sequence and molecular profile, and

recommended a course of action as a stopgap until, they hoped, mice could be created. (The company no longer offers expert panels.) Alan began that chemotherapy, which appeared somewhat successful. Doctors were then able to remove some of his tumor and implant it in avatar mice.

Ultimately, Robyn says, two regimens had a very modest effect in the mice, but none was aggressively killing off the cancer. One of those two was what the expert panel had already recommended. By the time the mouse results began streaming in, he was hours from death. Even a perfect avatar is only as good as the drugs available, and in Alan's case, none was good enough, at least not for the mice. "You want an answer," Sidransky says. "If the answer is no"—nothing works—"it's sobering, but it's still an answer."

Robyn feels the same way. She believes the initial analysis and treatment recommendations coordinated by the company helped extend her husband's life by perhaps 6 months. The mice ravaged by his cancer despite a barrage of drugs confirmed, for her, that there was nothing else they could have done. "We left no stones unturned," she says. "My kids and I can sleep at night. I don't ever wonder, 'If only.'" She has spoken out in support of Champions and now runs a website called CancerHAWK to connect families fighting cancer with resources that can help them.

To those who question whether Champions should be selling its promise, Robyn has a ready response. "It would really frustrate me as a patient advocate if there was a technology that might help" and yet was inaccessible, she says. "No matter what it costs—let me get my hands on it." ■

"[People are] selling a promise to patients before that promise has been judged to be true by really rigorous data."

George Demetri, Dana-Farber Cancer Institute

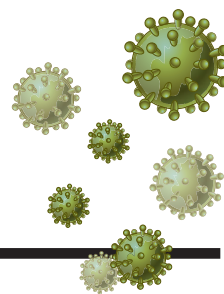
women suffer. All four approved therapies for ovarian cancer are tested on the avatars, and Haluska offers the most successful to a patient if her cancer resurges. "I need to prove that this is the way to go," he says, "that doing the avatars is better than standard" therapy.

One scientific shortcoming of PDX mice is that the animals lack a functioning immune system. This precludes trying immunologic therapies; it may also give misleading information about how drugs will behave in a person. Today's PDX mice, Hidalgo says, are "version 1.0." Several labs are developing and testing mice with a "humanized" immune system, but widespread use may be a year or more off.

Champions has run into other problems, too. Like anyone working with PDX mice, the company is unable to build avatars for everyone, and fails 30% of the time. For example, some tumor types, such as certain breast cancers and prostate cancer, don't readily engraft.

Of the patients who get a mouse, "about half choose to go further" into the drug testing phase, Morris says. Most who don't are bedeviled by time. In fact relatively few patients with advanced cancer survive long enough to use avatars as a road map, as critics of Champions are quick to point out.

"If you are selling ... to patients the idea of an avatar that could benefit their



PERSPECTIVES



Wingsuit diver Gleb Vorevodin jumps from the top of the Tianmen Mountain, China. Like this wingsuit diver, highly excited molecules can explore peaks and valleys in their energy landscape as they lose energy to form products.

PHOTOCHEMISTRY

Hot molecules—off the beaten path

Highly excited carbon dioxide dissociates unexpectedly to form molecular oxygen

By Arthur G. Suits^{1,2} and David H. Parker²

Much as a weary hiker would take the most level path to avoid unnecessary exertion, chemical reactions are generally assumed to proceed from reactants to products along the “minimum energy path” (MEP) through the potential energy surface (PES) that describes the barriers encountered as bonds break and form. In recent years, however, theoretical and experimental advances have allowed us to see more deeply into the dynamics of energized molecules, and there

is growing recognition that a highly excited molecule is more like a wingsuit diver who sails over the PES, roaming to new valleys and sometimes landing in unexpected terrain (1). Such non-MEP dynamics may well be the rule rather than the exception for hot molecules. On page 61 of this issue, Lu *et al.* (2) illustrate this point in the observation of an unexpected dissociation pathway to O₂ from isolated, highly excited CO₂ molecules.

Chemical intuition suggests that absorption of a high-energy photon by the very stable CO₂ molecule would never give rise to direct “photosynthesis” of molecular

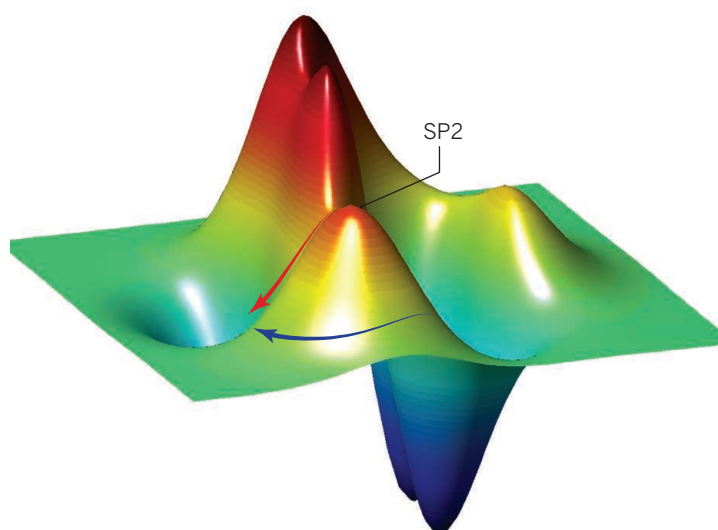
oxygen and a carbon atom. The expected molecular product, CO (formed by breaking either C=O bond), possesses a much more stable diatomic bond than O₂ (nearly double the binding energy). However, Lu *et al.* show that O₂ production occurred when CO₂ was exposed to extreme vacuum ultraviolet (VUV) light. Could this be the trigger for the “Great Oxidation Event” that turned Earth into a living planet? Probably not, but any formation channel—even a minor one (5% in this case)—could be important in tracing the history of Earth’s atmosphere and for understanding planetary atmo-

spheres and interstellar photochemical processes.

These CO₂ results may be an example of roaming, a particularly striking class of non-MEP reactions that has emerged in recent years, in which an excited molecule begins to dissociate by simple bond fission, but instead, an intramolecular reaction takes place that leads to unexpected products (3). A notable recent example has been the dissociation of NO₃, which was shown to exhibit roaming dynamics both in the ground state and in an electronically excited state (4). The CO₂ results of Lu *et al.* may be seen in a similar light. Although the mechanism is far too complex to be traced out in full detail, it seems likely that exotic configurations, such as a linear COO geometry, become accessible by launching the molecule through a metastable Rydberg state (one with highly excited valence electrons) at high energy. This step is followed by roaming or other non-MEP dynamics in the web of excited electronic states that leads to the unexpected C+O₂ dissociation pathway.

Recent theoretical work has suggested a condition for many of these non-MEP processes is a “second-order saddle point” on the potential energy surface, i.e., a hilltop separating two mountain passes (see the figure) (5). Reactants that find their way over these two passes will undergo very different forces and dynamics and emerge with distinct products or product distributions. The importance of roaming is that such features on the PES landscape appear almost universally in the vicinity of simple bond fission processes in molecules. Although seen and documented clearly in small molecules, related dynamics are suspected to play a role in glasses, metal clusters, and even protein folding (5).

These indications that highly energized molecules react in surprising ways have come from experimental advances, such as velocity-map ion imaging, that permit detection of the nascent velocity distributions of reaction products in select quantum states with extraordinary velocity resolution (6) and laser developments that push the envelope of excitation energy and pulse duration. To bring these methods to bear on the challenging CO₂ molecule, Lu *et al.* generated two sources of powerful tunable VUV radiation,



Exploring the valley. This schematic potential energy surface illustrates two distinct trajectories separated by a second-order saddle point. Potential energy is plotted versus two internal molecular coordinates; many additional dimensions are not shown. In the work of Lu *et al.* that explores CO₂ dissociation, molecular coordinates could include the C–O bond lengths and the O–C–O bond angle.

one for excitation and the other for product detection using imaging. In addition to these key experimental advances, state-of-the-art theoretical methods allow rigorous experiments to be conducted *in silico*, affording the intuitive insights of classical dynamics on an underlying foundation of high-level quantum theory (3). At the same time, advances in transition state theory and chemical kinetics strive to encompass the practical implications of these observations in compact and predictive models (7).

Experimental and theoretical methods continue to develop for application to highly excited molecules. Higher-energy photons are becoming increasingly available for photolysis studies, from table-top laser sources to third-generation synchrotron sources and free-electron lasers. Combined with ultra-detailed product detection schemes such as time-sliced velocity-map imaging or hydrogen-atom Rydberg tagging (8), important new details of the reactivity of molecules at high energies will continue to emerge. High-harmonic generation, for example, now can be used as an interferometric probe of the electronic and nuclear dynamics in excited molecules with subatomic spatial resolution and femtosecond time resolution (9).

Current efforts seek to exploit these and related methods to study dynamics in excited molecules on the attosecond time scale with pump-probe methods (10). These time scales correspond to that of electron motion in molecules. To gain access to these short time scales, the uncertainty principle requires a large energy bandwidth; such studies necessarily require radiation in the

extreme ultraviolet or soft-x-ray region of the spectrum. Advanced theoretical dynamics methods now allow us to solve the Schrödinger equation “on the fly,” even for systems involving highly excited electronic states (11). Efficient codes and faster machines will quickly see application of these methods to larger systems and more complex molecules.

Further advances in understanding the dynamics of more complex molecules and excited states will require experimental methods that can bring the needed level of spectroscopic detail. For larger molecules, this quickly becomes an insurmountable challenge given the usual laser-based methods. A promising alternative has recently emerged as the powerful chirped-pulse microwave technique is now being harnessed to perform investigations of photochemistry and reaction dynamics (12). Ironically, other avenues to the detailed investigation of energized molecules may come from ultracold experiments. Experimental methods to slow and cool molecules and ions for study yield reactants with a negligible smearing of the starting conditions, ideal for investigating the dynamics at the highest level of detail. We can be sure these new tools will bring new insights and be confident that excited molecules will offer more exciting surprises. ■

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GEOPHYSICS

Seafloor secrets revealed

Satellite data reveal formerly unknown tectonic structures

By **Cheinway Hwang¹** and
Emmy T. Y. Chang²

The trenches and ridges on Earth's seafloor are shaped by tectonic processes such as seafloor spreading and plate subduction. Detailed knowledge of seafloor tectonics is lacking in many areas. The most comprehensive data come from satellite altimeters, which use the strength and waveform of the radar signal returned from the sea surface to determine the tectonic properties of the underlying seafloor. On page 65 of this issue, Sandwell *et al.* (1) present the latest global marine gravity and depth data from altimeter missions CryoSat-2 and Jason-1. The data reveal buried tectonic structures, for example, in the Gulf of Mexico and the South Atlantic Ocean, that help to elucidate past tectonic processes.

The small-scale underwater tectonic structures seen in the gravity field of Sandwell *et al.* are particularly pronounced. This is because the gravity signal is strengthened by decreasing distance and these structures originate largely from shallow layers. Because of a major improvement in accuracy, this new gravity field will lead to more discoveries of tectonic features, especially in regions with thick sediments where sediment-induced gravity signals obscure tectonic structure-induced gravity signatures.

The vertical gravity gradient—that is, the change in gravity in the vertical direction from the surface—further enhances short-wavelength features and can be used to detect edges of geological transitions such as the continent-ocean boundary, where the properties of the rocks change from those of a continent to those of an ocean basin. Existing magnetic and seismic data cannot always resolve these features and have, for example, not been able to delineate fully the continent-ocean boundary in the northern South China Sea (2). A clear trace of this boundary, as well as features of an extinct spreading

center in the South China Sea, can be seen in Sandwell *et al.*'s data (see the figure).

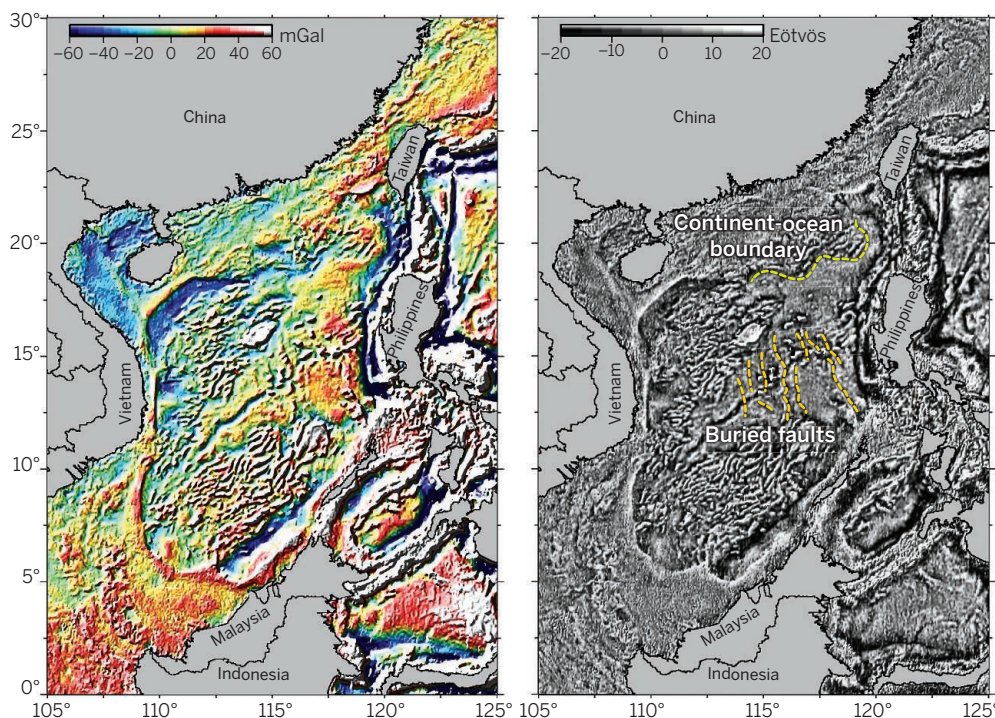
A method called waveform retracking is used to refine the radar return time and thereby improve altimeter ranging accuracy. The retracking techniques used by Sandwell *et al.* are designed to improve the accuracy of sea surface slopes determined from altimetry data (1, 3). Accurate and efficient methods are also needed to improve the accuracy of absolute sea surface heights (SSHs) from altimetry data; such data will improve identifications of oceanic signatures at medium to long wavelengths, such as mesoscale oceanic eddies and superswells (4, 5). The DTU global gravity grids (6) are based on retracking aimed at improving absolute SSHs. Neither retracking method may be able to fully decouple the contributing sources to radar measurements (range, wind, and wave height); further improvements may come from advanced techniques called knowledge-based iterative retracking and batch-jobbed multiple-waveform retracking that are under development.

To achieve optimal accuracy, it is important to combine satellite missions of varying

orbit inclinations to obtain a nearly equal accuracy for the north and east components of sea surface slope for gravity derivation (1, 7, 8). Currently, there are four high-resolution altimeter data sets to serve this need: Geosat, ERS-1, Jason-1, and CryoSat-2, with inclinations ranging from 66° to 108°. However, one must be careful in assigning appropriate weightings to each data set by considering their spatial resolutions and accuracies. This can be accomplished through iterative estimation of relative covariance factors between data sets.

The 1- to 2-mGal gravity accuracies achieved by Sandwell *et al.* (1, 7) are based on worldwide comparisons between altimeter gravity and high-quality ship gravity at wavelengths between 12 and 40 km. A gravity measurement accuracy of 1 mGal requires 1-mm accuracy in SSH measurements over 1 km along satellite ground tracks. This accuracy is a very challenging goal for measurement technology and data processing alike, especially in coastal areas and large inland water bodies such as the Caspian Sea, the Black Sea, and the Great Lakes.

The challenge in shallow-water regions arises from two factors: The waveform of the altimeter is distorted ("contaminated") by land mass, biasing the radar arrival time, and poor measurement corrections obscure the gravity signal (4, 6). For example, altimeter corrections in shallow waters for the effects of ocean tide, wet tropospheric delay, and



Hidden features of the seafloor. The marine gravity anomaly (left) and vertical gravity gradient (VGG) maps from satellite altimetry in the China Seas and the seas around southeast Asia reported by Sandwell *et al.* (1) show a wealth of tectonic features. The VGG image provides evidence for the continent-ocean boundary and the buried faults across the extinct spreading center.

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wave height have large uncertainties relative to such measurements made in the open ocean. Furthermore, SSH or slope cannot be converted to gravity at a coastal point unless land gravity is also known. Efficient extraction of tectonic features from the increasing data volume of altimetry will require novel data-processing strategies and gravity recovery methods (9, 10).

In addition to geophysical studies, altimeter gravity is increasingly important for coastal terrain mapping on land and at sea with technologies such as GPS, LIDAR (light detection and ranging), and satellite imaging. These applications require a highly accurate model of Earth's level surface (geoid) from gravity measurements. A dedicated, small ship-based coastal gravity survey can deliver 1-mGal accuracy at 500-m spatial resolution (11), but the cost is high. If 1-mGal altimeter gravity accuracy can be achieved at this spatial resolution, coastal nations, especially at lower latitudes, will no longer need shipborne or airborne gravity measuring campaigns for purposes such as resource exploration and coastline topography determination.

Sandwell *et al.*'s results are a breakthrough in space-based marine gravity observation. The key factors driving this success are advances in altimeter technology (10, 12), an improved processing technique (3, 7), and a dedicated algorithm for deriving gravity and depth from altimetry (1, 8). As CryoSat-2 continues to increase the coverage of satellite ground tracks to densify spatial coverage and several innovative altimeters are planned for launch (10), we will soon be able to detect even finer-scale gravity signatures that can benefit studies ranging from marine resource exploration to tectonic evolution. ■

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The hunt is on. Cheetahs reach famously high speeds during hunting, but Scantlebury *et al.* show that it is the search for prey rather than the chase itself that is energetically more costly.

ECOLOGY

How large predators manage the cost of hunting

For pumas and cheetahs, seeking prey is more energetically costly than the subsequent chase

By John W. Laundré

Being a large carnivore is not easy. First, there is the food, the energy they need to survive, which by definition consists mainly of other animals. This means that meeting daily energetic needs is not as easy as just going out and gathering plants that are waiting around to be found and eaten. Large carnivores often prey on animals that are bigger than themselves and that try to avoid being killed. Foraging by carnivores becomes a two-player game of stealth and fear (1), making it more difficult and thus energetically costly for carnivores to catch enough to stay alive. Large carnivores must balance the energy spent seeking and subduing prey with the energy they get back when they catch something—which does not happen as often as one might think (2–4). Two reports in this issue, by Scantlebury *et al.* (5) on page 79 and by Williams *et al.* (6) on page 81, look at how two carnivores, cheetahs (*Acinonyx jubatus*; see the first photo)

and pumas (*Puma concolor*; see the second photo), tread the fine line of energy losses and gains in order to survive.

The carnivores investigated in the two studies seek prey in very different ways. Pumas are sit-and-wait hunters, whereas cheetahs typically chase their prey at high speeds. The results of the studies should thus help to elucidate the effect of energetic demand on hunting style.

There have been ample studies of the energetics of carnivores. However, most attempts to calculate the energetics of large carnivores have not explicitly determined the specific energy necessary for seeking and subduing prey. Most have relied on estimates of metabolic rates under laboratory conditions (7, 8) or velocities and distances traveled over 24 hours based on telemetry or Global Positioning System data gathered from wild animals

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with collars (9, 10). Williams *et al.* and Scantlebury *et al.* instead used more specialized techniques to measure specifically the energy that a carnivore expends during the critical phases of searching for and subduing prey. The most ingenious was the use by Williams *et al.* of a new SMART (species movement, acceleration, and radio tracking) collar that differentiated among various activities such as resting, eating, and running. The results help to understand how the two species have evolved to compensate for these high-energy-demand activities and have consequences for the conservation of these species.

Both studies concur that seeking prey is much more energetically costly than subduing the prey. Intuitively, one would think that the extreme physical exertion of chasing and pouncing on prey would far outweigh the searching process. As both studies point out, however, although subduing prey is energy-intensive, the time spent doing this is short. Carnivores either quickly capture their prey or give up. Furthermore, Williams *et al.* found that pumas adjust their energy investment based on prey size, not overexerting themselves on smaller prey. Of interest, but not addressed in these articles, is whether carnivores take into consideration the amount of energy they are willing to expend for a capture before giving up on the

“Pumas are sit-and-wait hunters, whereas cheetahs typically chase their prey at high speeds. The results of the studies should thus help to elucidate the effect of energetic demand on hunting style.”

chase. If they do, could their current energetic state, that is, the time since they last ate, have an effect on their behavior? Will they end a chase sooner or be less willing to attempt subduing more energy-intensive prey depending on how long ago they have last eaten? Such data would increase our knowledge of whether carnivores are regulating their energy expenditures even more than these articles suggest.

Because seeking prey is the more energy-intensive activity, both studies propose that these species have evolved ways of reducing this demand. Pumas can substantially reduce the energy demand of seeking prey with their sit-and-wait approach, as do most felids—ex-

cept cheetahs. Cheetahs, and probably other carnivores that chase their prey, opt for minimizing the time they are mobile and seeking prey. Scantlebury *et al.* point out that kleptoparasitism—the theft of prey by other animals, in this case by lions and hyenas—increases how often or how long carnivores such as cheetahs have to seek animals. However, they note that if prey abundance is high, cheetahs can quickly find additional animals to pursue, reducing the impact of kleptoparasitism to an acceptable level.

The overall conclusion of the two studies is that evolution has honed the behavior of these two carnivores to maintain the balance of energy needed in finding and subduing a reluctant food source. Both authors caution, however, that human activity can disrupt this balance. Any human-caused loss of vegetative cover may make it harder for a sit-and-wait predator to sneak up on its prey. Human-reduced prey abundance would require predators to expend more energy in the costly search for prey to hunt. In either case, these changes can increase the energy that predators expend in both seeking and subduing prey, and thus threaten their survival. In this case, conservation of adequate habitat and prey abundance become paramount in efforts to conserve large carnivores.

The two studies greatly increase our understanding of the energetic demands of large carnivores, but there is still more to do. The ecological concept of the landscape of fear, where prey alter their habitat use based on predator lethality, has a flipside for the predator, the landscape of opportunity (11). In this model, in some habitats, predators have an easier time capturing prey (12), thus requiring less energy expenditure. Incorporating how carnivores use their landscape of opportunity to minimize energy expenditures for seeking and subduing their prey will go even further to help us understand how large carnivores manage and manipulate their role in the cat-and-mouse game of life. ■

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Stealthy hunter. Pumas hunt by stalking and ambushing their prey. Williams *et al.* show that—as in the case of the cheetah—seeking prey is more energetically costly than subduing the prey.

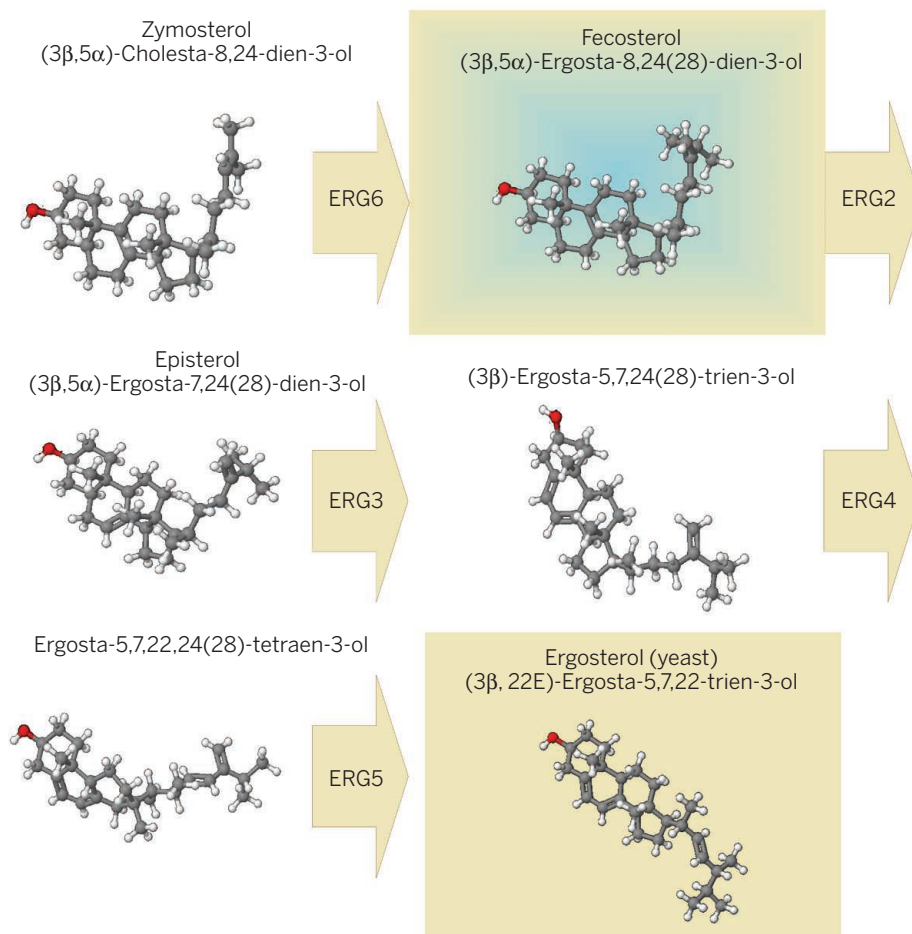
How to survive being hot and inebriated

Cell membrane integrity and composition are key to making yeast a better biocatalyst

By Clint Cheng and Katy C. Kao

Industrial biotechnology will play an increasingly important role in our response to climate change and the need for increased environmental sustainability. In the realm of biofuels production, baker's yeast (*Saccharomyces cerevisiae*) is often the biocatalyst of choice. Sustainable bio-based chemical production requires the efficient use of renewable feedstock such as lignocellulosic biomass. However, a major challenge in this field is the wide variety of inhibitory conditions encountered by the biocatalyst throughout the production process. Two papers on pages 75 and 71 of this issue address this challenge, using two distinct approaches to advance the current state of the field. Caspeta *et al.* (1) made use of adaptive laboratory evolution to generate strains conferring stable thermotolerance. They employed genomic, transcriptomic, and metabolic flux analysis tools to elucidate the underlying mechanisms for the observed phenotype. Lam *et al.* (2) hypothesize a mechanism of product toxicity in the generation of ethanol and propose a route for achieving increased ethanol tolerance.

Primary sources of inhibitory stress encountered in industrial biocatalysis include the high concentration of desired products (e.g., ethanol), undesirable by-products, high operating temperatures, and suboptimal pH levels. In the case of biofuel production, high titer and productivity of ethanol are necessary for economic viability; thus, tolerance to high concentrations of ethanol is among the most desired phenotypes. In addition, tolerance to thermal stress has recently become a particularly useful phenotype as the industry moves toward the use of SSF [simultaneous saccharification (the breaking down of cellulose and hemicellulose to hexose and pentoses) and fermentation], in which biomass hydrolysis and microbial fermentation are carried out in a single step. The use of SSF can lower operating time and costs (3); however, the optimal temperature of the hydrolyzing enzymes is generally higher than that allowing for the viability of *S. cerevisiae*. Although strains of yeast more tolerant to these stressors have been isolated, the design of robust biocatalysts remains a challenge,



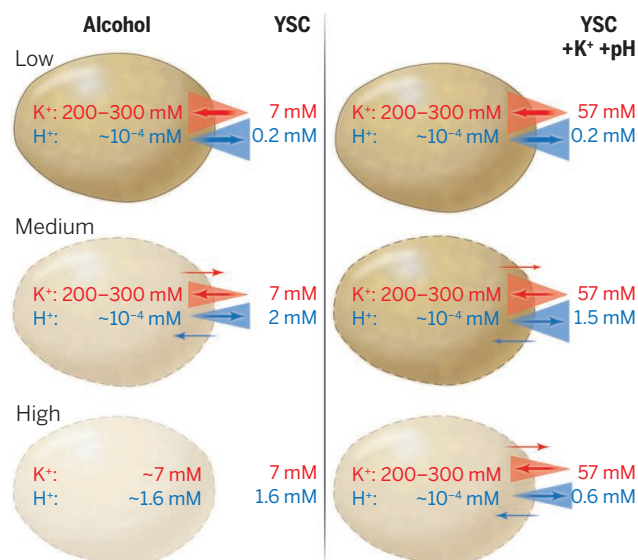
Some yeast can withstand the heat. Modification in sterol composition leads to enhanced thermotolerance in yeast. Enzyme-catalyzed steps (ERG2 to ERG6) on the ergosterol biosynthesis pathway illustrate the structures of fecosterol and ergosterol (1).

and a lack of available information on the mechanisms underlying these traits impedes progress in the field.

Numerous methods have been used to generate thermotolerant *S. cerevisiae* strains. Analyses of gene expression profiles of strains exposed to short, nonlethal heat shocks have led to the identification of many genes involved in the organisms' response to thermal stress (4). These induced responses, however, do not represent changes at the genome level, limiting the ability to use the data in potential reverse-engineering efforts. Alternatively, genes from naturally thermophilic organisms have been cloned into *S. cerevisiae* to improve thermotolerance; for example, thermotolerant yeast have been engineered by introducing ubiquitin and heat shock pro-

tein genes from the thermophilic archaea *Thermoanaerobacter tengcongensis* (5).

Caspeta *et al.* have taken a more global approach based on adaptive evolution in the laboratory. They selected for thermotolerant mutants by prolonged propagation of *S. cerevisiae* through serial batch transfers at the elevated temperature of 39.5°C (baker's yeast normally grows best at 30°C) until a substantial improvement in population-level growth rate was observed. Detailed molecular characterization of isolated individual clones from the evolved populations revealed that all isolates analyzed contained nonsense mutations (mutations that encode a premature stop codon) in the *ERG3* gene. To elucidate the effects of the *ERG3* mutation, they introduced the point mutation in *ERG3* identified in TT11,



Learning to handle alcohol. A proposed mechanism by which modification of ionic gradients enhances ethanol tolerance in yeast (2). At low alcohol levels (**top**), potassium and proton gradients across the cell membrane remain high. Increases in alcohol levels permeabilize the cell membrane, allowing ions to freely diffuse (**bottom left**). Increases in extracellular potassium concentration and pH (**right**) reduce leakage by lowering concentration differences in and out of the cell (2).

one of the evolved thermotolerant mutants, into wild-type yeast. The resulting strain, M7, was able to grow considerably faster than the wild-type strain at 40°C, confirming that this mutation indeed confers thermotolerance. *ERG3* is purported to encode a C-5 sterol desaturase, an enzyme involved in an intermediate step in the ergosterol synthesis pathway (6), ergosterol being a component of fungal cell membranes. To explore the possible role of *ERG3* in thermotolerance, the authors analyzed the sterol composition of M7, TT11, and wild-type yeast. The total sterol contents present in the three strains were similar, whereas their sterol compositions were not. The wild-type yeast contained primarily ergosterol, while M7 and TT11 contained primarily fecosterol, which is an intermediate of the ergosterol pathway.

Ethanol toxicity in yeast is in part mediated by disruption of the yeast cell membrane (7). Lam *et al.* used this information to hypothesize that modification of ion composition in the culture medium could be used to mitigate the effects of toxicity. Indeed, they found that the coupling of increased extracellular potassium ion concentration with a decrease in proton concentration provided a means to increase ethanol tolerance. More important, they demonstrated that the same modification in electrochemical gradient also confers tolerance to isopropanol and isobutanol, suggesting a broad applicability of this strategy to other potential biofuels. They used these results to rationally engineer laboratory strains with increased expression of plasma membrane proton exporter PMA1 and potas-

sium importer TRK1, leading to increased performance in ethanol production equivalent to industrial strains without the need for media modifications.

Caspeta *et al.* illustrate the power of combining evolutionary engineering with a multi-omics approach for inverse strain engineering of a complex phenotype, implicating *ERG3* in thermotolerance. Deletions in the ergosterol pathway, including that of *ERG3*, have previously been shown to decrease membrane rigidity (8). As the work of Lam *et al.* also suggests that the plasma membrane is critical in ethanol tolerance, further characterization of membrane structure and integrity in the respective tolerant strains is needed to determine the exact physiological effects of increasing fecosterol and reducing ergosterol as well as altering electrochemical gradients in the cell.

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PHYSIOLOGY

Good barriers make good neighbors

Cerebrospinal fluid may provide indicators of age-associated decline in brain function

By Richard M. Ransohoff

The human brain exists in a unique environment, shielded from the blood and conditioned by its own remarkably active metabolism, yet dependent on the blood-brain interface to import nutrients and remove waste. Age-related decline in brain function is almost certainly caused by multiplexed pathways that incorporate intrinsic brain failure, worsening systemic environment, and accumulation of toxic metabolic waste. If we could identify and monitor alterations in an organ that reflects the determinants of age-associated decline, we might better understand how these changes arise and perhaps even counteract them. On page 89 in this issue, Baruch *et al.* (1) have done just that. They studied gene expression in a series of organs from young and old mice. The authors propose that the choroid plexus—a specialized structure lying deep within the brain—may provide a readout of the brain's overall status and may potentially mediate some facets of debility during aging.

The choroid plexus is a relatively obscure organ suspended in the brain's cerebral ventricles, large cavities filled with cerebrospinal fluid (see the figure). The plexus consists of a monolayer of cuboidal epithelial cells wrapped around a dense vascular stroma with unusually large capillaries and arterial elements that lack smooth muscle. This epithelial-stromal composite is organized in extensive fingerlike villi. The choroid (chorionlike) plexus, named by analogy to the heavily vascularized stroma in the eye (2), also contains both macrophages and dendritic cells.

Macromolecules in the blood have limited access to cerebrospinal fluid (and vice versa) because of a blood-cerebrospinal fluid barrier that is established by the choroid plexus (3). The cerebrospinal fluid is critical for delivering growth and differentiation factors to the developing brain (4), but with adulthood,

ILLUSTRATION: P. HUEY/SCIENCE

this function lessens. Biomedical interest comes largely because the interstitial fluid of brain equilibrates with cerebrospinal fluid, whose composition yields invaluable clues to biological or disease processes. The barrier function of the choroid plexus is critical for healthy brain development and is present from early embryogenesis, carried out by primitive neuroepithelial cells even before its formation (5). The other canonical function of this organ has become controversial after 100 years of consensus: The choroid plexus is no longer considered the sole producer of cerebrospinal fluid in the adult brain. Instead, most of the cerebrospinal fluid may arise from meningeal vessels, which traverse the protective membranes covering the brain (6). Importantly, epithelial cells of the choroid plexus are continuously exposed both to the peripheral environment, represented by the stromal vasculature, and to the central nervous system milieu, in the form of the cerebrospinal fluid.

Baruch *et al.* sought to determine which organs in the aging mouse might harbor gene expression changes that could help identify interior environmental factors that underlie loss of function in the aging brain. The authors concentrated on organs of the immune system (spleen, thymus, lymph nodes) and on organs communicating with the exterior (gut, lung, liver). They also studied two sites in the central nervous system: hippocampus (a portion of the brain associated with learning and memory) and choroid plexus. Using next-generation RNA sequencing, they characterized all transcribed genes at early adulthood (3 months of age) and old age (22 months).

This massive data set was analyzed using the Gene Ontology resource, which harnesses manually and electronically curated databases to parse individual genes as being associated with biological processes or molecular function. Gene Ontology comparisons between young and old mice and among the organs surveyed showed increased age-dependent transcription of genes associated with immune function in all organs exam-

ined. This finding does not specify a particular immune response or process, only that genes reported to be present during immune functions show elevated expression in older as compared with younger mice. Notably, the transcription of genes in the type I interferon (IFN-I) pathway was elevated in the choroid plexus of older compared with younger animals, and much more strongly in this organ than in other sites. Several genes associated with a related cytokine, IFN-II, decreased in the choroid plexus with age.

Baruch *et al.* examined whether age-related changes in the brain or the blood were affecting transcription in the choroid plexus. Mice were joined together surgically so that

suggest that imbalances in *IFN-I/II* transcription emerge over time in the choroid plexus and affect brain function adversely.

A notable strength of the study by Baruch *et al.* is its focus on the choroid plexus as a unique organ exposed to blood (stromal side) and cerebrospinal fluid (brain side). The clever use of parabiosis discriminates between influences from these two compartments, and the study indicates that some aspects of cognitive decline with age might be associated with the brain's environment (substances in blood or cerebrospinal fluid) rather than brain-cell failure.

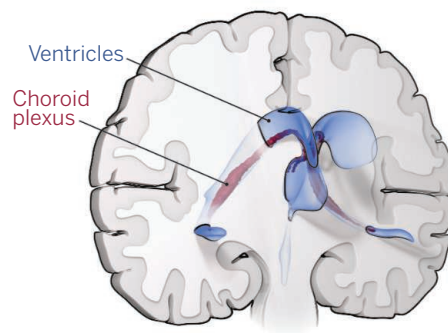
The study is limited, however, by the lack of convincing evidence for a specific imbalance of signaling by choroid plexus IFN-I versus IFN-II as a pathogenic element for age-related brain failure (7). It will be important to analyze the specific genes in the Gene Ontology categories to determine if there are alternative explanations for the changes observed (for example, action of another cytokine or products of damaged cells), because the expression of stimulus-sensitive genes can be induced readily by varied signals. For example, transcription of one *IFN-I* signature gene analyzed by Baruch *et al.* was also triggered by treating cells in vitro with a cytokine mixture of interleukin-1 β , tumor necrosis factor- α , and interleukin-6. Another example is *Cxcl10*, which the authors reference as an IFN-II-regulated gene that declines with age in the choroid plexus. In fact, *Cxcl10* transcription can be readily induced either by IFN-I or IFN-II signaling (8, 9) or can also be IFN-I and IFN-II independent (10). Expanding and sharpening the data analysis are essential to ensure that the proper molecular modulator(s) of brain function are being targeted for therapy.

Another consideration is that the choroid plexus may actually produce only a very small fraction of cerebrospinal fluid, with most coming through bidirectional flux across cerebral vasculature (whose surface area is almost unimaginably huge) (6). If this newer concept of cerebrospinal fluid production and flux has merit, then the choroid plexus may be a remarkably useful mirror of environmental influences on the brain without producing sufficient factors by itself to modulate brain function. ■

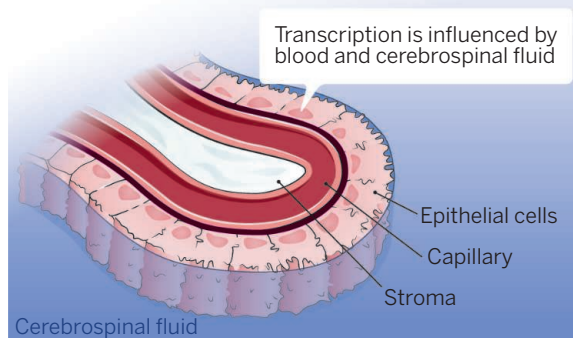
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Coronal section through the human brain



Section through choroid plexus



Influences on the brain. The choroid plexus produces some cerebrospinal fluid and constitutes the blood–cerebrospinal fluid barrier. The choroid plexus is exposed both to blood and cerebrospinal fluid, so its gene expression reflects both blood and brain components, which may modulate brain function.

they shared a single circulation (parabiosis); that is, a young and old mouse were engineered to share blood. Increased IFN-I pathway signaling in the choroid plexus could not be induced in young mice by blood-derived factors from old mice. This indicated that age-related increase in *IFN-I* transcription in the choroid plexus arose from influences coming from the brain via the interstitial fluid and cerebrospinal fluid. Transiently modifying IFN-I signaling using a blocking antibody increased learning in older mice. In addition, Baruch *et al.* observed that mice lacking the IFN-II receptor showed a modest age-related impairment in learning. The data

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The unstoppable oil palm? Oil palm is a tropical crop that presents the highest yield per hectare of oil-producing crops in the world. New high-yielding varieties could spare land for forests worldwide or could increase tropical deforestation as oil production is transferred to the tropics. The image shows land cleared for oil palm plantation in Malaysian Borneo.

CONSERVATION

A double-edged sword for tropical forests

Contrary to expectation, high-yielding tropical crops may cause forest loss in the tropics

By **L. R. Carrasco**,¹ **C. Larrosa**,^{1,2}
E. J. Milner-Gulland,² **D. P. Edwards**³

With a growing global population and increasing per capita consumption, reconciling agricultural production with biodiversity conservation is a major challenge to humanity (1). A frequently promoted solution to stem the tide of agricultural expansion is to increase crop yields, allowing global demand to be met without further tropical forest losses. Recent genome sequencing of key crops such as oil palm, eucalyptus, rubber, soybean, rice, and cocoa could facilitate substantial yield increases (2–4). Could such yield improvements offer a solution to both tropical forest loss and agricultural demand, or could they pose further challenges to tropical conservation?

Among the above crops, oil palm is one of the main drivers of tropical forest conversion (see the first figure) as a result of increasing demand for cosmetics, edible oil, and biofu-

els. As global demand for oil palm continues to grow (5), there is concern that the massive forest conversion to oil palm observed in Southeast Asia (see the second figure) could be reproduced in Africa and Latin America.

Oil palm yield increases are already a reality. For instance, top producer Sime Darby's new "super palms" variety, which can increase yields from 4 to 10 tons/ha and was developed through molecular breeding, is expected to debut next year (6). Increasing yields are aligned with the Malaysian government's oil palm strategy of replacing low-yield palms with new higher-yielding varieties. Oil palm genome sequencing may also make possible the generation of seeds resistant to diseases, drought, and salinity. As a result, currently low-yielding areas would become more productive.

Intensification through yield improvement is at first glance very positive news for conservation. In theory, the global demand for palm oil could be met on less land, thus sparing land for nature (7). By 2050, the demand for palm oil is expected to rise substantially, leading to dramatic additional demand for land in the absence of yield improvements (5). Moderate yield

improvements, from the current 4 tons/ha to 5.2 tons/ha, could lead to a global saving of 7 million ha (47% of the current global oil palm area or 7% of Indonesia's forests) that would be converted under the status quo assumptions (5). However, these estimates do not account for international trade and the fact that palm oil can be substituted by buyers for other vegetable oils such as soybean or rapeseed oil.

Simulations with a global trade general equilibrium model suggest that an assumed 56% increase in oil palm yield per tree in Malaysia and Indonesia has two potentially positive outcomes for biodiversity: a drop in the global price of palm oil by 4.3% and of vegetable oils generally by 2.5%, and a net increase in global forest area of ~300,000 ha (8). The simulations suggest that ~400,000 ha of agricultural land (equivalent to ~6% of Indonesia's oil palm area) would be taken out of production in Brazil, India, and Canada. Left uncultivated, this land could regenerate into secondary forest of conservation value.

The simulations in (8) also show a potential negative outcome for biodiversity: an expansion of 65,000 ha of cropland for



CONSERVATION SERIES

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oil palm and 47,000 ha for pastures at the expense of tropical forests in Malaysia and Indonesia. The reason is that technological advances lead to higher returns to farmers in the region, encouraging the use of more land (8). Thus, a substantial proportion of the potential for tropical forest regeneration is negated by market effects.

A large part of the land sparing with potential for forest regeneration occurs in temperate regions, where most species are not threatened with extinction. For example, 40% of the predicted spared land is in Canada (8), presumably because high-yielding oil produced in tropical countries outcompetes crops such as rapeseed or soybean grown in temperate regions. This transfer of vegetable oil production from temperate to tropical regions as a result of international market mechanisms (9) is a worrying consequence of improving oil palm yields.

As the demand for palm oil continues to increase, yield improvements could also present further challenges to conservation by

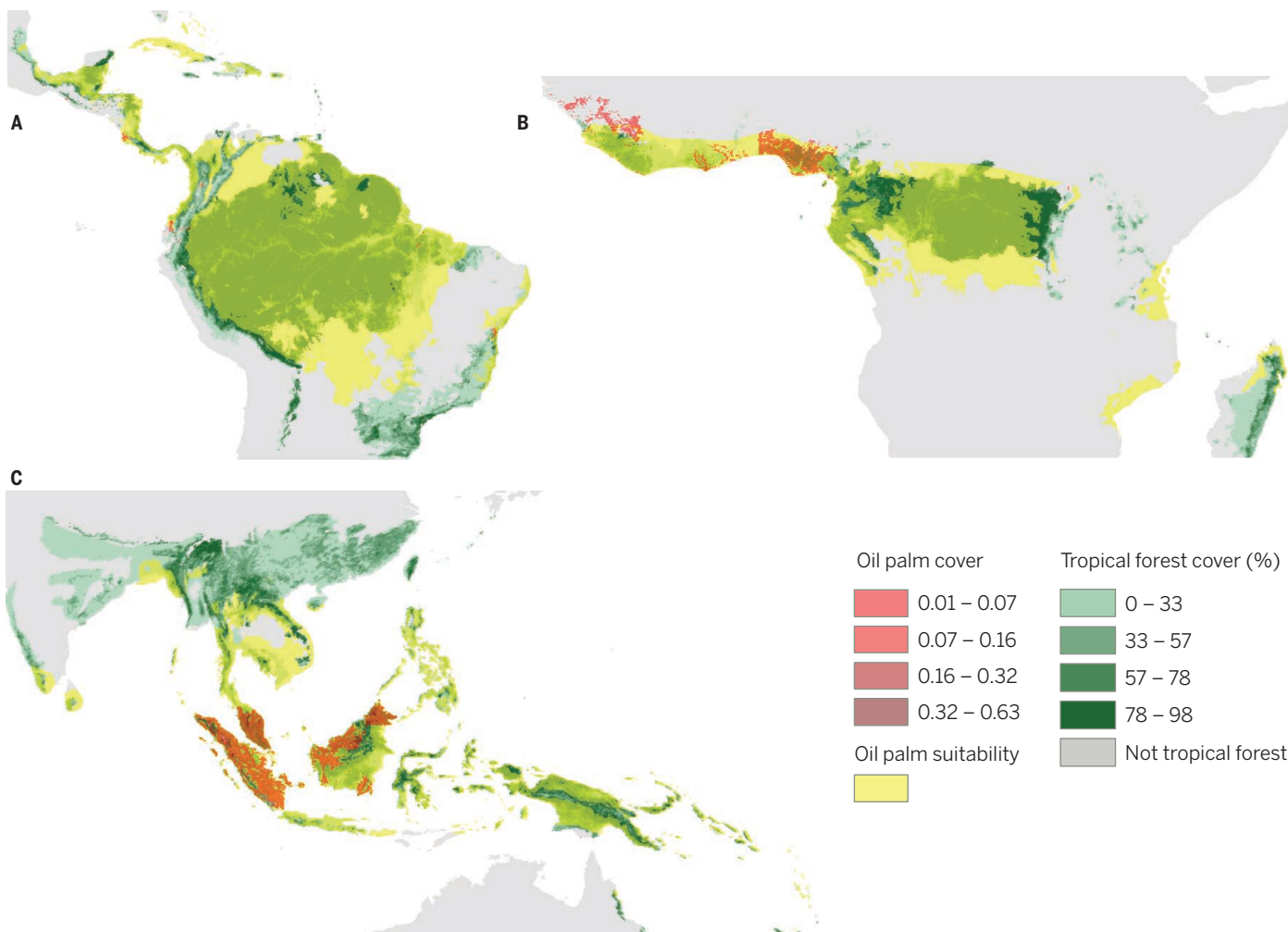
allowing expansion of oil palm to countries where little oil palm is currently grown. In both Africa and Latin America, vast tracts of land highly suitable for oil palm production are still covered by tropical forests (see the second figure). Lower yields of African and Latin American varieties and higher production costs have so far prevented an explosive expansion of oil palm production in these regions. Having exploited the most suitable land for oil palm in Southeast Asia, oil palm companies are now investing in Africa (10). Availability of high-yielding varieties more suitable for difficult growing conditions is likely to accelerate this trend.

The global interconnectedness of oil palm production requires a large-scale, predictive approach to conservation planning (7). For instance, African governments could control the expansion of new oil palm varieties by dividing land into areas available for oil palm conversion (such as degraded land), those that are key for local livelihoods and require community-level management, and those al-

located mainly for conservation. Landscape-scale planning could help to reconcile the economic benefits of the new varieties with ecosystem service provision (such as flood protection and carbon sequestration), improving the livelihoods of local people, and minimizing biodiversity losses.

Global initiatives can also help to realize the potential social and conservation benefits of land spared through oil palm yield improvements. Such initiatives may include the introduction of strong safeguards by governments to prevent further expansion of oil palm cropland into forests and into communal agricultural areas important for local food security. Certification schemes such as the Roundtable for Sustainable Palm Oil (RSPO) should be strengthened through the inclusion of stricter environmental criteria and greater consumer pressure for change. This would give companies an incentive to respond to biodiversity concerns (10).

The case of oil palm resonates with other crops driving tropical deforestation



Rapid expansion. Oil palm has been one of the main drivers of deforestation in the tropical forests of Southeast Asia. New high-yielding varieties could promote expansion in Africa and Latin America by increasing its productivity there. The maps overlay extant tropical forests, in 2005, distribution of oil palm covering more than 1% of the landscape in 2000 (14), and oil palm yield potential > 0 [based on oil palm suitability maps from (15)] in (A) Latin America, (B) Africa, and (C) Southeast Asia.

for which genetic improvements have recently been achieved. For instance, new high-yielding genetically modified varieties of eucalyptus are being considered for use by Brazil, raising concerns that the new varieties will encourage further expansion of plantations and pose biosafety risks through gene flow (11). Although the oil palm varieties under current consideration are not genetically modified, common patterns exist in their implications for tropical land use. Genome sequencing and variety improvement are also likely to affect the land use dynamics of many other tropical crops. A consortium that includes Mars Inc. and the University of California has sequenced the cacao genome and is working on sequencing almost 100 other neglected African crops (12). The new varieties could be capable of pest and disease resistance, drought tolerance, and higher yield quality. These new traits could improve livelihoods but may also lead to crop expansion in previously unsuitable areas.

As the oil palm example shows, there is a danger that improvements in tropical crop yields will further transfer agricultural production from temperate to tropical regions, leading to more tropical deforestation and thereby limiting the social and ecological benefits of high-yielding varieties. To help governments to prepare for the indirect effects of the new crop varieties, scientists need to relate globally interconnected land use dynamics to local conservation actions (13). This requires the development of models of the drivers of environmental change that incorporate feedbacks at a range of scales. These models will help governments to preempt and plan for the unintended negative consequences of technical advances. ■

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IMMUNOLOGY

A neighborhood watch upholds local immune protection

T cells that reside in peripheral tissue create conditions that enable local and broad responses to infection

By Francis R. Carbone and Thomas Gebhardt

"Memory" T cells are "antigen-experienced" cells that are generated during a prior infection. Capable of migrating through extra-lymphoid regions of the body (1), they are poised to mount a swift and strong response when they next recognize the pathogen. The description of effector memory T cells (T_{EM}) built on this notion (2), and by extension, peripheral immunity was thought to rely extensively on T_{EM} cell recruitment from blood to extra-lymphoid tissues such as the gut and skin. Nevertheless, recent attention has turned to memory T cells that permanently reside in the periphery, with little or no representation in the wider circulation. These "tissue-resident" memory T cells (T_{RM}) are more effective in controlling peripheral infection than their circulating counterparts (3). On pages 93, 98, and 101 of this issue, Iijima and Iwasaki (4), Schenkel *et al.* (5), and Ariotti *et al.* (6), respectively, probe the mechanistic underpinnings of the persistence and protective function of tissue-resident memory T cells.

Whereas most studies on tissue-resident memory cells have featured the CD8 T_{RM} cell subtype, Iijima and Iwasaki examine the CD4 T_{RM} cells remaining in mouse mucosa after the clearance of vaginal infection with herpes simplex virus. Those CD4 T_{RM} cells were not replenished from the blood and do not freely migrate. Instead, they were found in aggregates surrounding macrophages, forming a distinct microanatomical structure that the authors call a "memory lymphocyte cluster." The main driver of cluster formation appears to be a combination of the cytokine interferon- γ (IFN- γ), which is released by the CD4 T_{RM} cells, and chemokines [predominantly chemokine (C-C motif) ligand 5] produced by macrophages that are central to cluster formation (see the figure). Importantly, ablation of the macrophages or disruption of the chemokine network resulted in dissolution of clusters, disappearance of CD4 T_{RM} cells, and concomitant loss of local immune protection.

What drives the IFN- γ expression in memory lymphocyte clusters is left undefined, although Iijima and Iwasaki speculate that residual antigen may be involved despite the absence of detectable virus. Although CD4 T_{RM} cells and CD8 T_{RM} cells share important commonalities, there are key differences that support this idea. Both subsets demonstrate parallel modulation of molecules required for tissue egress, such

"Tissue-resident memory T cells may be the immune cells of choice in designing strategies to stop pathogens before they establish a meaningful infection."

as the sphingosine-1-phosphate receptor 1 and C-C chemokine receptor type 7 (7, 8). However, memory lymphocyte clusters are not required for CD8 T_{RM} cell retention, especially at body surfaces where these cells can be widely dispersed within the epithelium (9, 10). More importantly, CD8 T_{RM} cells can form and persist in the absence of prior localized infection (11). Differences in the requirements for long-term peripheral persistence between CD4 T_{RM} cells and CD8 T_{RM} cells were highlighted in an earlier study that showed that only CD8 T_{RM} cells remained in vaginal mucosa after nonspecific recruitment (12). By contrast, their CD4 counterparts were not maintained under those same conditions, now explained by the absence of memory cluster formation without prior infection (4). Whether this reflects an underlying antigen-dependent basis for CD4 T_{RM} cell retention, in contrast to a cell-intrinsic CD8 T_{RM} developmental process (7, 8), remains unknown.

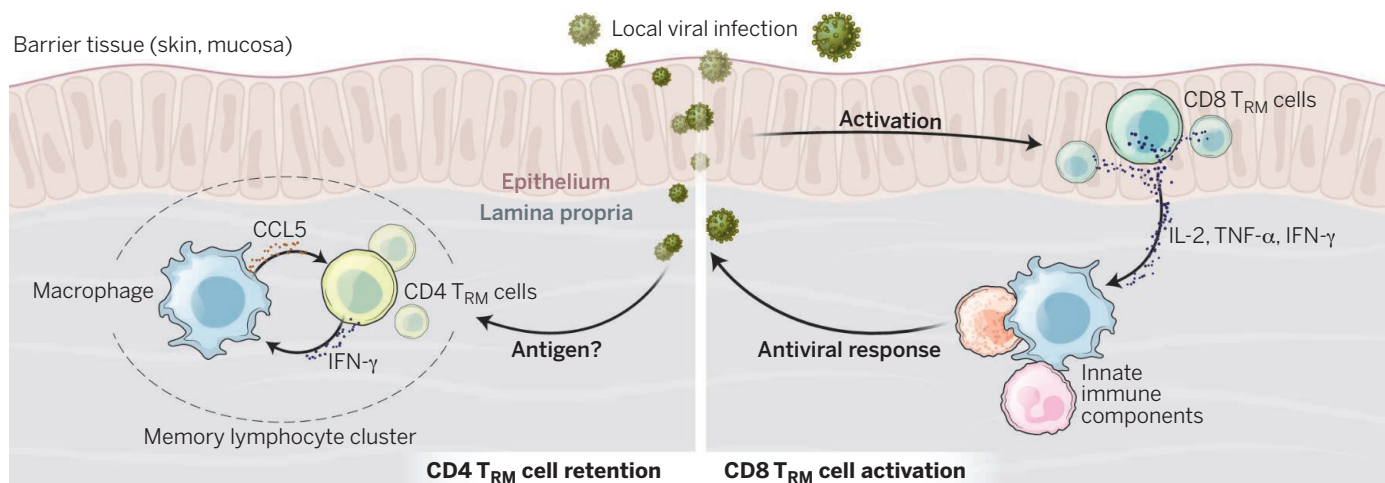
Schenkel *et al.* and Ariotti *et al.* focus on the effector mechanisms involved in infection control by CD8 T_{RM} cells. A rapid CD8 T_{RM} cell-dependent recruitment of circulating memory T cells to sites of viral infection has been described as a consequence of im-

mediate release of IFN- γ by CD8 T_{RM} cells, brought on by local antigen stimulation (13). Schenkel *et al.* show, in mouse models of lymphocytic choriomeningitis virus and vaccinia virus infection, that this early cytokine production has a wider impact. Upon pathogen encounter in the female reproductive tract, CD8 T_{RM} cells produced a range of cytokines that rapidly promoted lymphocyte recruitment by increasing the expression of endothelial vascular cell adhesion molecule-1, in addition to activating local innate immune cells (dendritic cells and natural killer cells). Thus, the CD8 T_{RM} cells act as early sensors of infection with strikingly broad “alarming functions.”

dramatically demonstrated by Ariotti *et al.* They examined gene transcription in skin that either contained or was devoid of embedded virus-specific CD8 T_{RM} cells. Intuitively, one might have expected differences in T cell-related transcripts to dominate in response to viral challenge, given the presence or absence of CD8 T_{RM} cells. Instead, the differences overwhelmingly involved genes associated with classical innate immune responses, with the majority driven by IFN- γ . For example, expression of the *Ifitm3* gene, which encodes the antiviral product interferon-induced transmembrane protein 3, was induced within hours of CD8 T_{RM} cell activation. Similar to Schenkel *et al.*,

traditional direct cytolysis of infected cells, as the latter requires high effector-to-target ratios for efficient protection. Furthermore, tissue conditioning was evident immediately after engagement of the tissue-resident memory T cells, even in the absence of their circulating counterparts.

Given this ability to rapidly generate an almost tissue-wide antiviral state, tissue-resident memory T cells are perfectly poised to inhibit the first rounds of pathogen replication. Such immediate action is likely critical for effective protection against pathogens such as HIV, which rely on a wave of replication at the site of invasion to feed downstream reservoirs of persisting infection that



Curb your infection. (Left) Resident CD8 T_{RM} cells are dispersed within the epithelium of a barrier tissue (such as skin or vaginal mucosa), whereas CD4 T_{RM} cells aggregate in memory lymphocyte clusters. Cluster maintenance relies on constitutive IFN- γ production by CD4 T_{RM} cells, which may be linked to persisting viral antigens, and the consecutive production of CCL5 by macrophages. **(Right)** Local immunity by CD8 T_{RM} cells is mediated by their production of cytokines such as IFN- γ , tumor necrosis factor- α (TNF- α), and interleukin-2 (IL-2), upon restimulation by virus. These cytokines act on various tissue and immune cells, triggering innate immune responses and thereby inducing a nonspecific antiviral state that is sufficient to protect even from unrelated bystander infections.

Early sensing of infection has traditionally been viewed as a property of the innate immune system, whereas Schenkel *et al.* show that pathogen recognition has one key hallmark of adaptive immunity—antigen peptide specificity and thus memory of prior infection. Consequently, specific antigen sensing by CD8 T_{RM} cells results in widespread recruitment of innate immune components. In an impressive demonstration of this cross-over between innate and adaptive elements, Schenkel *et al.* show bystander protection against an irrelevant infection, an effect not typically associated with the exquisite specificity of adaptive immune components such as the CD8 T_{RM} cells that are central to this effect.

The induction of an organ-wide antiviral state by tissue-resident memory T cells is

Ariotti *et al.* demonstrate that this CD8 T_{RM}-driven innate immune activation provides immediate protection from infection, even if challenged with a virus unrelated to the original lodgement specificity.

A key commonality between the studies of Iijima and Iwasaki, Schenkel *et al.*, and Ariotti *et al.*, is the ability of tissue-resident memory T cells to profoundly curb the course of infection in barrier tissues. Of note, infection control by both CD4 T_{RM} cells and CD8 T_{RM} cells involves their local production of IFN- γ , which then acts on a broad variety of tissue cells. There is also a clear conditioning of the local environs by the immediate engagement of innate immune elements, and this is central to wider protection against infection. Ariotti *et al.* point out that relatively few CD8 T_{RM} cells can activate the immune milieu around the site of viral challenge, amplifying a small adaptive signal to a potent state of broad immune protection. This amplification has advantages over

are virtually impossible to eliminate (14). Tissue-resident memory T cells may be the immune cells of choice in designing strategies to stop pathogens before they establish a meaningful infection. ■

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PSYCHOLOGY AND ECONOMICS

Progress in measuring subjective well-being

Moving toward national indicators and policy evaluations

By Alan B. Krueger^{1,2*} and
Arthur A. Stone³

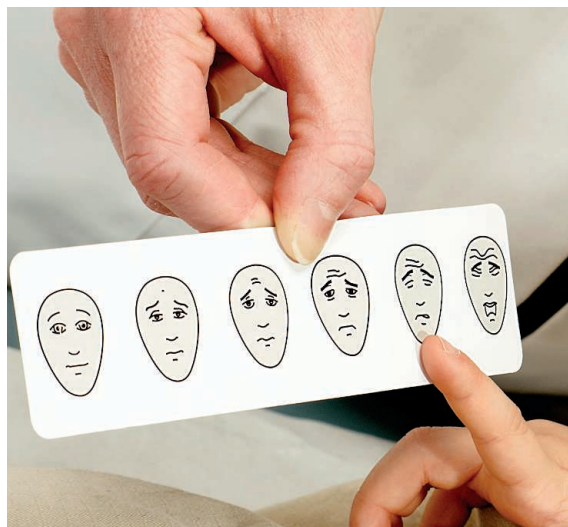
Progress in science requires new tools for measuring phenomena previously believed unmeasurable, as well as conceptual frameworks for interpreting such measurements.

There has been much progress on both fronts in the measurement of subjective well-being (SWB), which “refers to how people experience and evaluate their lives and specific domains and activities in their lives” (1). In 2009, the Sarkozy Commission recommended adding SWB measures as supplements to existing indicators of societal progress such as gross domestic product (GDP). In light

POLICY of subsequent activity by governments and international organizations, we summarize several important advances and highlight key remaining methodological challenges that must be addressed to develop a credible national indicator of SWB and to incorporate SWB into official statistics and policy decisions.

VALIDITY AND COMPARABILITY. Subjective well-being is necessarily measured by respondents’ self-reports evaluating their life and feelings (see the photo). In some fields, such reports are the standard, e.g., assessment of pain and fatigue. Although there have been long-standing concerns about the meaning of subjective reports, emerging evidence finds that self-reports are related to biological processes and health outcomes, which increases confidence in the validity of such measures. Experimental studies find that self-reports of the extent of pain are associated with changes in blood flow to brain regions known to process pain (2). Studies have shown correspondence between subjective reports of affect and experience with immunological and hormonal measures (3, 4). Mortality has been associated with low levels of SWB (5).

Nevertheless, additional evidence is needed to support the validity of between-group comparisons, e.g., when comparing SWB across countries or demographic groups. Differential reporting styles, including interpretation of questions and response



Faces Pain Scale—Revised [©2001, International Association for the Study of Pain]

scales, by groups, has the potential to yield misleading conclusions. Some rankings of countries have been questioned on these grounds.

A recent technique for evaluating and adjusting interpersonal and intergroup differences in self-reports is the “vignette approach.” Questions about the constructs under investigation are preceded by vignettes that describe an individual at some intensity of the construct, providing an explicit comparison standard for the subsequent rating. Responses can be compared and scales adjusted if systematic intergroup differences emerge (6). This has proved promising for assessing subjective health appraisals and is being applied to well-being reports, although there are potential environmental factors that may confound comparison, such as differences in the quality of health care across communities.

Interpretation and use of response scales (i.e., options for answering questions) is likely to vary according to past experiences, cultural background, genetic factors, and immediate context. Well-known response options are verbal scales (e.g., “Extremely satisfied” through “not at all satisfied”) and numeric rating scales (e.g., 0 through 10 anchored scales, with 0 indicating the absence of some feeling). On the basis of studies of individual differences in densities of taste receptors on subjects’ tongues, Bartoshuk (7) described scale elasticity, wherein self-reports with standard response scales of

taste sensation were invalid. Response options had different meanings for two groups of tasters. “Supertasters” had a “stretched-out” scale, a feature lost in current response scales. The same logic may prove useful for SWB.

Another validity issue concerns the extent to which people adapt to their circumstances. There is a critical distinction in how scales are used, where extreme events can result in “recalibration” (8), as opposed to true adaptation. In the first case, changes in SWB are not due to actual differences in experience or evaluation but simply to how the scale was used. True adaptation is defined by changes

in emotional experience. There is evidence that people shift their use of scales and that they can adapt to circumstances; e.g., in response to physical disabilities or winning a lottery [see (9) for a recent meta-analysis]. But adaptation is not a uniform process, and some circumstances and aspects of SWB appear relatively more or less resistant to adaptation.

CONCEPTUAL FRAMEWORK. The conceptual framework for SWB remains, in our view, the most important obstacle to developing a comprehensive national indicator. Innovative new work (10) provides a framework in which individuals’ utility depends on several fundamental, nonoverlapping aspects, such as material well-being, life satisfaction, and emotional experience. Following standard economic logic, components can be aggregated on the basis of the extent to which individuals would be willing to trade an improvement in one aspect against an improvement in another, on the margin. This method is implemented by posing hypothetical choices between situations that involve different dimensions of SWB. These marginal valuations provide weights for aggregating aspects of SWB, just as prices (marginal utilities) provide appropriate weights for aggregating components of the GDP under certain assumptions.

This research is in its infancy, and objections can be raised about its reliance on revealed preference, but it provides a coher-

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ent framework for aggregating dimensions of SWB on the basis of individuals' choices. Further refinements—perhaps including actual, as opposed to hypothetical, choices—may advance the measurement of SWB.

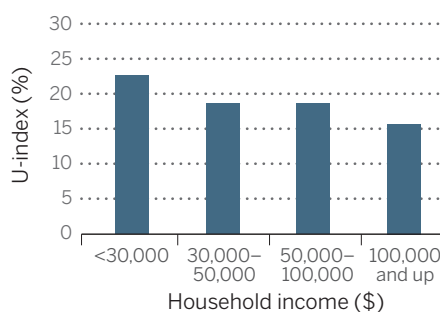
Kahneman and Krueger (11) seek to side-step many requirements for a comprehensive index of SWB (such as interpersonal comparability) by focusing only on experiential SWB and measuring the percentage of time that people spend in an unpleasant state, which they call the U-index. An unpleasant state is defined as an episode in which the intensity of a negative emotion is greater than the intensity of positive emotions (see the chart). They justify the U-index in part by arguing that policy-makers often care more about minimizing misery than maximizing happiness [a theme echoed in (1)]. The U-index can be constructed with the Day Reconstruction Measure, which collects time-use data together with emotional experience. The U-index is robust in that different individuals and groups can interpret scales differently, as long as they consistently apply their interpretation to positive and negative emotions. The U-index is related to, but conceptually distinct from, more traditional measures of affect (measured with momentary and diary approaches), in that the U-index emphasizes that one dominant-negative emotion can color an entire episode or day.

Until more progress is made toward developing a credible, comprehensive index of SWB, we would emphasize the importance of separately measuring the key components of SWB (e.g., satisfaction with life, positive emotional experience, meaning in life) and keeping them distinctive.

OFFICIAL MEASURES. After the Sarkozy report, the UK Office of National Statistics (ONS) initiated a program to measure SWB in its Annual Population Survey. Evaluative (“satisfaction” with life), eudaimonic (well-being or human flourishing), and hedonic (affect in everyday life) SWB were surveyed. However, the assessment consists of only four questions and lacks information on actual time use or events in people's lives. To partly address these concerns, ONS plans more detailed surveys of SWB. The proper way to combine ONS's measures into a comprehensive indicator is unresolved.

The Organization for Economic Cooperation and Development (OECD) published an extensive report on measuring SWB (12) to guide national statistical offices and presented a detailed critique of ongoing efforts. The OECD's Better Life Index finesses the problem of how to aggregate different components of well-being by allowing users to set their own weights. The OECD has as-

U-index by household income



U-index. The proportion of time that a rating of sad, stressed, or pain exceeds happy. [Data from (17)]

sembled a “high-level” commission to analyze topics that could be informed by SWB research, such as income inequality, and the United Nations launched initiatives on well-being and sustainability (e.g., the International Day of Happiness).

Earlier this year, the National Research Council (NRC) of the U.S. National Academy of Sciences issued a report on hedonic well-being and policy (1). This stressed the importance of considering both happiness and misery in SWB. It supported a broader definition of hedonic well-being (called experiential WB) that includes pain and other forms of suffering, which the panel considered important for policy purposes. The U.S. Bureau of Labor Statistics has incorporated an affective module in the American Time Use Survey (ATUS) in 2010 and 2012 to combine SWB data (happy, pain, sad, stress, tired, meaningful) with time-use information during representative periods of the day. The NRC report highlighted that, “The ATUS SWB module is practical, stable, inexpensive, and worth continuing.... Not only does the ATUS SWB module support research; it also generates information to help refine SWB measures that may be considered for future additions to official statistics” (1).

A striking feature of the OECD and NRC reports is their optimism about future prospects of SWB measures. Another recent report on well-being and policy stated “we should measure well-being more often and do so comprehensively.... This would help governments improve policies, companies raise productivity, and people live more satisfying lives” [(13) executive summary].

POLICY EVALUATION. Measures of SWB have become key outcome measures in program evaluation, often yielding deeper insights than traditional measures. For example, 10 to 15 years after the launch of the Moving to Opportunities experiment, which randomly offered some low-income families

living in U.S. public housing the opportunity to move to less-disadvantaged neighborhoods, significant improvements were found for components of SWB (distress, depression, anxiety, and calmness) but not for economic (employment and earnings) and educational outcomes (14). Evaluations of the Oregon Medicaid expansion found significant improvements in subjective outcomes, including depression and self-reported health, but mixed results concerning physical health (15). Because individuals and policy-makers value subjective outcomes and because such outcomes appear to be affected by major policy interventions, measures of SWB are likely to play an increasingly important role in policy evaluation (16) and decisions.

Further advances in measurement and the conceptual framework for combining SWB measures are needed before sufficient consensus can be reached to support a comprehensive, official measure of SWB that can be compared at the national level. In the meantime, comparisons of various components of SWB, with a focus on negative emotional experiences, strike us as a reasonable agenda for national statistical agencies and researchers. ■

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A. B. K. was a member of the 2009 Sarkozy Commission and the ONS Forum on Measuring National Well-Being. A. A. S. chaired the National Academy of Science's Panel on Measuring Subjective Well-Being in a Policy-Relevant Framework. A. B. K. and A. A. S. are members of the OECD High-Level Expert Group on Multilevel Subjective Well-Being. Financial support from the National Institute on Aging Roybal grant P30AG024928 and grants R01AG042407, R01AG0406629, and P01AG05842 are gratefully acknowledged.

BOOKS *et al.*

SOCIOLOGY

Risk and responsibility

By **Bridget M. Hutter**

Awareness of financial, technological, physical, and terrorist disasters across the globe is high as modern communication systems relay details and graphic footage into our homes. Yet the solutions seem remote, as if they can only be handled and understood by specialists and experts and not by us. Kathleen Tierney very effectively disabuses us of this in a powerfully written account of how all types of disaster are socially produced, that is, the result of social processes and decision-making. She carefully constructs her case using familiar social science literatures and a wide range of examples of disasters from different geographical regions. It is good to see work on risks and disasters coming together, because so often they are considered apart. The transatlantic divide on these topics is also partially overcome by using the work of European theorists to set the scene about the relationship between risk, modern societies, and technological risks. Tierney skillfully and critically pushes the boundaries of her arguments to embrace the social production of natural risks. A popular example would be climate change, socially produced and exacerbated by a collective failure to control its causes.

Decision-making is crucial to the social production of risk. It takes place at different levels of social living, from social cognition, through organizational, social, structural, and ultimately global levels of decision-making. Navigating through these levels and the complex theories that help us to understand the social roots and channels of risk is difficult. We need to grasp the parameters that shape what we see and know, for example, cultural assumptions that inform our prioritization of goals and our ability to envision worst-case scenarios. There may be institutional blindness and organizational barriers

to collective sense-making and risk management. The literatures on these subjects are large, scattered, and multidisciplinary and brought together admirably in this work.

A strong theme of the book is inequality: why are some communities and countries much more severely affected by disasters than other communities experiencing similar events? Power is part of the answer: the power to define, frame, and prioritize risks. Economic power is regarded as paramount, especially the power to sanction rapid urbanization and the concentration of populations alongside large industrial sites in areas of natural hazard. Here, we have a clear example of the social production of risk as the result of decision-making that has unequal, socially structured effects: they render the poor, elderly and migrant communities particularly vulnerable.

There are also significant inequalities between nations, where developed nations tend to experience the greatest monetary loss from disasters and less-developed countries tend to suffer the greatest loss of life.

Just as risks are socially constructed, so are their solutions. The main focus of this work is "resilience," a relatively new concept in which a great deal of faith has been placed. Resilience refers to the ability to absorb sys-



Rethinking our roles in preparing for and responding to catastrophic events.

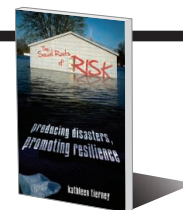
tem shocks and to cope, adapt, and bounce back from disruption. This can entail major reorganizations at different social levels from the individual to the household, community, and economy. Tierney shares faith in resilience but is careful to explain the difficulties in implementing this approach. Her discussion of the concept is comprehensive, cutting through multiple literatures to identify its core characteristics. How to achieve resilience is the bottom-line question and one she tackles head on. She explores sources of resilience and how it might be assessed and promoted. Notions of robustness, redundancy, resourcefulness, and rapidity emerge as important. These allow the strength and flexibility necessary to promote resilience. "Social capital" emerges as a precondition for developing and maintaining other forms of

**The Social Roots of Risk
Producing Disasters,
Promoting Resilience**

Kathleen Tierney

Stanford Business Books,

2014. 317 pp.



capital that might help overcome vulnerability in the event of a disaster. Cooperation and social contacts within and between groups can enhance resilience and help to access wider networks and resources.

Resilience planning is not about abandoning predisaster planning but having the flexibility to adapt plans or knowing when to put them aside. This may involve new emergent groups, especially networks that appear much more resilient than hierarchies: They can tap into diverse sources of information and resources and build on pre-existing social capital. Predisaster planning enhances adaptive capacity.

Policy recommendations flow from this. For instance, civil society groups often take the lead in disasters, and they can be helped to be better prepared to enhance community resilience. Governance and regulation are also important. They are embedded in

the social, economic, and political structures that produce them. Tierney recognizes this in relation to the ability to resist regulation, but the sections on capture are less convincing. The forces that weaken regulators are much more subtle and complex than the notion of capture conveys. Regulators often work in political and legal conditions that prevent them from doing their job. There are moments of resistance, most often in the wake of disasters, such as the new confidence

in regulation that has emerged in the wake of the financial crisis. It may be helpful to coopt and embrace a mix of state and non-state regulation, combining civil and even economic regulation to help in the transformational change Tierney envisages but sees as unlikely to take effect.

This book has high and perhaps unrealistic aspirations, but it is important reading. It makes a convincing case about why natural and social science disciplines need to work together toward risk and disaster reduction. In doing so, we need to be mindful of some of the danger of expecting that we can fully anticipate and manage risks, allowing space for resilience and unorthodox approaches to develop.

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LETTERS

Edited by Jennifer Sills



Global collaboration

In your experience, what is the biggest challenge to global scientific collaboration? How should it be addressed? In July, we asked young scientists to tell us their thoughts.

NEXTGEN VOICES

A sample of their responses can be found below. To allow for as many voices as possible, in some cases we have printed excerpts of longer submissions (indicated by ellipses) and lightly copyedited original text for clarity. To read the complete versions, as well as many more, go to <http://scim.ag/NG12Results>. Follow Science's NextGen VOICES survey on Twitter with the hashtag #NextGenSci.



...IN MANY CASES, scientific research and the consumer market that can potentially benefit the most are separated by cultural and political boundaries.... From my experience in the medical device industry, collaboration is hindered by researchers' inability to distinguish between the various attitudes toward death in indigenous versus modernized cultures. It is important for me as a scientist to understand which parts of the world have greater demand for the most technologically advanced life-saver. Greater cultural

competency must be engrained in scientists' brains from a young age, and it is best learned through experience, not from a textbook. Thus, I propose that the scientific community reevaluates its outlook on study abroad programs to encourage aspiring scientists to learn overseas....When young researchers make breakthroughs in their respective fields, they should be able to quickly identify which parts of the world they should collaborate with so that they can help the people who need their innovation the most.

Nirupa Galagedera

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SINCE 2011, I'VE been regularly involved in global mental health collaborations between India and the United States. I initiated these because I am originally from India and moved to the United States for

graduate school....A big challenge I have faced is allowing my Indian research collaborators to have increased interaction with my team in the United States. I go to India once a year to transfer my knowledge and train collaborators, but it would really be great to have more mechanisms that allow my collaborators and mentees to visit me in the United States often, perhaps for internships of 3 to 12 months, to learn firsthand from our methods and then go back and apply these in their local setting. If such travel and interaction were built into global science collaborative grants, and more such grants were available, they would be bound to stimulate greater global collaboration....

Jyoti Mishra

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THE OLDEST AND still the biggest barriers to global scientific collaboration are religious and cultural differences....The problem can be addressed only by removing the borders between countries. People should be able to travel all around the world freely (without applying for a visa), know new cultures, share food, and share how to create value for humanity. This process will surely take a long time and involve many security and public issues. However,...if we want to discover new planets, first we need to learn to live together in the world.

Meryem Ayas

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I BELIEVE THAT the current system, in which research is mostly funded and organized at the national level,...is the greatest barrier to global scientific collaboration. We need a global research system, in which every country, developed or developing, must contribute to a central fund that seeks to establish research infrastructure across the globe....The United Nations was established to take care of global security; the same should be done for scientific research....

Patrick Kobina Arthur

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...BUILDING TRUST between researchers in different parts of the world and sustaining it throughout the project is undoubtedly the biggest challenge facing global collaboration....After practicing research in Egypt and the United States, I can say that different countries manage collaboration differently due to many factors, including cultural variations and differences in working environment. These differences might lead to misunderstanding between collaborators, a gap in connection, and consequently an easy loss of trust, which results in problems that hinder progress and allow more problems to develop. Scientists worldwide should set rules and

ethics for effective collaboration; a global norm would provide a common ground for scientists to build together the trust required for the project to succeed. I also suggest having a course in each graduate school for young scientists...to learn these rules and ethics and get the chance to apply them early in their careers.

Islam Mosa

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...THE BIGGEST challenge to global scientific collaboration is the lack of funding earmarked for the costs of collaboration itself....Funding the exchange of knowledge itself—and not a tangible thing like a piece of equipment or a stipend for a worker—does not seem to be on the radar of most funding agencies. Worse, there is an unrealistic expectation that expertise—like data—could or should somehow be exchanged online rather than in person....Despite the tantalizing idea of scientific work crossing borders freely (as business does), we lack the funding infrastructure to maintain large-scale scientific collaboration on a global level.

P. William Hughes

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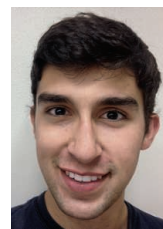


THE BIGGEST challenge that global scientific collaboration faces is dealing with technical secrets and issues of national security. I know of one collaboration that was forced to terminate midstream because the results could have, potentially, been used for military purposes, and this was considered a “security threat” to one of the countries involved. To make the case even worse, the foreign researcher was investigated and faced the risk of being deported from the primary country of the collaboration. Such collaborative work is not only a waste of money and time, but could be disastrous for the researcher's career and personal life. Therefore, I believe that before embarking on such research endeavors, all parties should understand the areas where collaboration is permitted and which lines should not be touched or crossed....It

would be very helpful if governments provided researchers with directions so they could avoid risky areas and thus protect themselves....

Lei Jiao

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THE BIGGEST challenge to global scientific collaboration is the language barrier....In my 6 years of struggling to learn French, I often found that Google Translate's abilities were improving faster than mine. Unfortunately, automated translation of technical terms and scientific papers lags by comparison. We should assemble a network of bilingual scientists to collaborate with Google; together, they will produce scientific software on par with Google Translate....

Sam Allon

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REGULATORY mandates are a major obstacle to global scientific collaboration. Research that is deemed permissible in one country may be illegal in another country....This barrier has been highly visible in the field of human embryonic stem cell research, where researchers in the United States must adhere to legislation and funding limitations that researchers in European countries have not faced. There are many reasons why regulations developed by individual countries are appropriate to govern the research occurring in that nation, but disparate regulations are a major obstacle for worldwide collaboration in the scientific community.

Kara Kuntz-Melcavage

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...IT WOULD BE wonderful to have free international scientific websites for every subject, where all groups could share their manuscripts, thesis papers, books, and opinions related to a

specific research topic. This would enable us to interact easily because, although international meetings are important, not everybody has the opportunity to attend them and they don't usually last for more than a week. Therefore, by creating networks, it would be possible to share information and ideas among scientific communities....

M. Romina Schiaffino

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THERE EXISTS a wide gap in the technological advancement among developed, developing, and undeveloped countries. To address any global problem, collaboration among all these countries is a

must. However, the divide that exists in technologies, working culture, requirements, and vested interests of the participating countries will hinder implementation of any global project....To tackle such problems, time and funding for preparation of common facilities at each collaborating unit should be provided before the project starts. Identification and recruitment of equally competent collaborating members should be handled seriously. Strict monitoring of project implementation progress should not be just limited on papers, but be physically verified at each location.

Poonam C. Singh

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...THE IMMIGRATION cap in many countries... and the issues associated with international mobility of research funds...affect science and research.

To enable scientists to tap into the global scientific networks and collaborations, it would be beneficial if universities and research centers were separated from any immigration cap. International mobility of R&D funds, if made more transparent and less bureaucratic, would also help the mobility of scientists and science, in general. Given the scale of issues that science tackles, governments should put more emphasis on facilitating technology

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Deadline for submissions is 14 November. A selection of the best responses will be published in the 2 January 2015 issue of *Science*. Submissions should be 100 words or less. Anonymous submissions will not be considered.

transfer to encourage global scientific collaborations.

Swati Negi

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IN MY OWN experience, diplomatic issues have lately put way too many obstacles before global scientific collaboration....I was born in Brazil, and I currently live in Israel. Israel could share

technology with Brazil in, at least, fields like surveillance and riot control, drought avoidance and water reservoirs management, and border control. Meanwhile, Brazil has a vast experience in exploring oil and gas in deep waters, a field that Israel is starting to work on. But relations between them were stressed due to diplomatic issues concerning the current military operation.... There should be scientific cooperation councils in every government whose main activity would be creating the proper conditions for a scientific collaboration even if the countries involved don't keep good (or any) diplomatic relations.

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FEAR IS THE cause of separation. PIs and Ph.D.s alike are most afraid of being scooped. That is why we are hiding locally and theme-wise in our scientific niche....

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THE BIGGEST challenge to global scientific collaboration is that funding dictates what should be the subject of research instead of identified problems motivating scientific objectives. In

most cases, when you bring together scientists from the developed world (mostly carrying the funding purse) and those from the developing world (existing with specific challenges), there is no proper challenge-mapping in identifying important research areas that have shared relevance. Often, researchers from developed countries brush aside input from scientists from the developing countries....What is needed are equal partnerships....

Collet Dandara

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THOUSANDS OF Fellows, most of whom are young investigators, visit or study in different countries under sponsorship by Chinese government scholarships each year. The biggest challenge in global

scientific collaboration for most of them is lack of ideas and innovations. Their supervisors or overseas collaborators are actually serving as idea/theory providers, while many of these investigators are learning new methods and designing the experiments to verify others' ideas and theories. There is an interesting saying that the laowai (foreign scientists) dig holes, but Chinese investigators fill them with papers,...

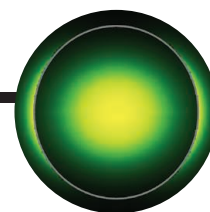
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RESEARCH

A single gold nanoparticle can control the direction of light

Petersen et al., p. 67



IN SCIENCE JOURNALS

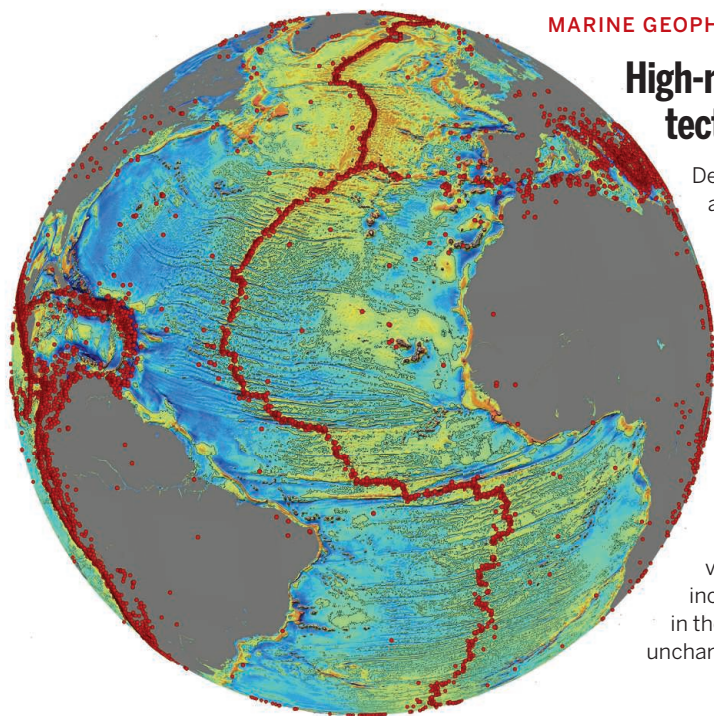
Edited by Stella Hurtley

MARINE GEOPHYSICS

High-resolution tectonic solutions

Detailed topographic maps are available for only a small fraction of the ocean floor, severely limited by the number of ship crossings. Global maps constructed using satellite-derived gravity data, in contrast, are limited in the size of features they can resolve. Sandwell *et al.* present a new marine gravity model that greatly improves this resolution (see the Perspective by Hwang and Chang). They identify several previously unknown tectonic features, including extinct spreading ridges in the Gulf of Mexico and numerous uncharted seamounts. — BG

Science, this issue p. 65; see also p. 32



helped prevent the decrease in cognitive function seen in aged mice. — LBR

Science, this issue p. 89; see also p. 37

T CELL MEMORY

Resident memory T cells sound the alarm

Immunological memory protects against reinfection. Resident memory T cells (T_{RM}) are long-lived and remain in the tissues where they first encountered a pathogen (see the Perspective by Carbone and Gebhardt). Schenkel *et al.* and Ariotti *et al.* found that $CD8^+ T_{RM}$ cells act like first responders in the female reproductive tissue or the skin of mice upon antigen-reencounter. By secreting inflammatory proteins, T_{RM} cells rapidly activated local immune cells to respond, so much so that they protected against infection with an unrelated pathogen. Iijima and Iwasaki found that $CD4^+ T_{RM}$ cells protected mice against reinfection with intravaginal herpes simplex virus 2. — KLM

Science, this issue p. 98, p. 101, p. 93; see also p. 40

MAMMALIAN ENERGETICS

The costs and benefits of stalking and chasing

Organisms live under a constant balance between getting and using energy. Large carnivores may feel this balance more acutely because of the large amounts of energy needed to capture and subdue their prey. Williams *et al.* and Scantlebury *et al.* used remote measures of physiology and behavior to identify the hunting strategies of the stalking North American puma and the speedy African cheetah (see the Perspective by Laundré). In both cases the cats' hunting strategies are well matched to produce a balance between the energy they spend on the hunt and the energy

they acquire from their prey, despite their very different strategies and levels of competition. — SNV

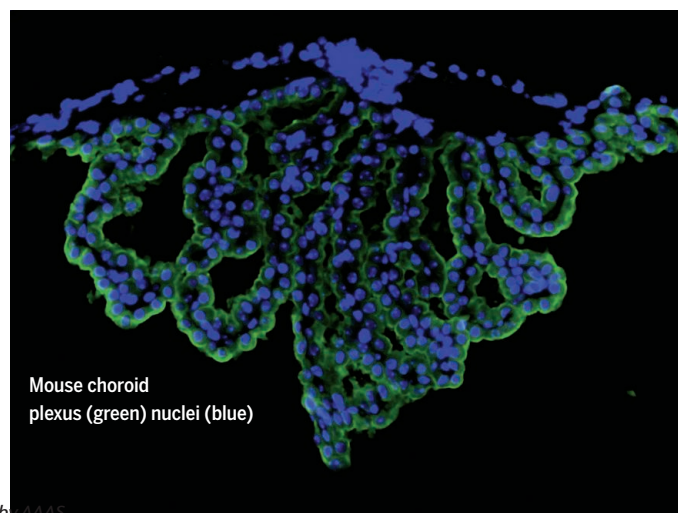
Science, this issue p. 81, p. 79; see also p. 33

AGING

Excess signaling is bad for the aging brain

Preventing antiviral-like responses may protect function in the aging brain. Baruch *et al.* monitored messenger RNA production in the choroid plexus, the interface between the blood and cerebrospinal fluid, in young and old mice (see the Perspective by Ransohoff). They detected an inflammatory response in older mice not

present in the brain of young mice that was also seen in old aged human samples postmortem. Preventing signaling by the cytokine interferon- β , which normally helps in the antiviral response of the immune system,



Mouse choroid plexus (green) nuclei (blue)

BIOFUELS

Tricks for boosting yeast's ethanol yields

To become a widely used source of fuel, widespread industrial production of ethanol using yeast needs to be simple and efficient. However, two conditions ideal for boosting production—tolerance of higher temperatures and high concentrations of ethanol—have been limiting (see the Perspective by Cheng and Kao). Now, Caspeta *et al.* have used adaptive laboratory evolution to find yeast strains that can tolerate high temperatures and Lam *et al.* have identified a route to improve yeast's resistance to high concentrations of ethanol. — NW

Science, this issue p. 75, p. 71; see also p. 35

HIV EPIDEMIOLOGY

The hidden history of the HIV pandemic

Rail and river transport in 1960s Congo, combined with the sexual revolution and changes in health care practices, primed the HIV pandemic. Faria *et al.* unpick the circumstances surrounding the ascendancy of HIV from its origins before 1920 in chimpanzee hunters in the Cameroon to amplification in Kinshasa. Around 1960, rail links promoted the spread of the virus to mining areas in southeastern Congo and beyond. Ultimately, HIV crossed the Atlantic in Haitian teachers returning home. From those early events, a pandemic was born. — CA

Science, this issue p. 56

PHOTOCHEMISTRY

Illuminating oxygen out of carbon dioxide

It has long been known that high-energy ultraviolet light can split carbon dioxide into CO and O fragments. Lu *et al.* have now uncovered a parallel pathway that appears to yield C and O₂ instead (see the Perspective by Suits and Parker). By precisely measuring the energy and trajectory of the carbon

fragment after CO₂ irradiation, O₂ formation could be inferred. The results introduce a potential mechanism for abiotic oxygen production in CO₂-heavy atmospheres of other planets. — JSY

Science, this issue p. 61; see also p. 30

ASTHMA

How the common cold can worsen asthma

Rhinoviruses—the main causes of the common cold—can make asthma attacks worse. Now Beale *et al.* report that one reason may be because rhinoviruses cause lung epithelial cells to make the cytokine interleukin-25 (IL-25). More IL-25 is produced in people with asthma than in those that are healthy. In mice with allergic “asthma,” rhinovirus infection triggered IL-25 production, and blocking the IL-25 receptor eased the increased asthma symptoms. Thus, as the cold season approaches, blocking IL-25 may be a promising therapeutic strategy in asthmatics. — ACC

Sci. Transl. Med. **6**, 256ra134 (2014).

CANCER

A new approach for treating colon cancer?

Most patients with colon cancer have a mutation that results in the Wnt/ β -catenin pathway being “on” all the time. But inhibitors of this pathway interfere with the continuous renewal of the epithelial cells lining the intestinal tract. Pesse *et al.* discovered that the signaling pathway involving the receptor gp130, its associated Jak kinases, and the transcription factor Stat3 enhanced the growth of intestinal tumors in mice. Inhibiting this pathway stopped cell proliferation and reduced tumor growth. Drugs targeting the Jak-Stat3 pathway are currently in clinical trials for treating hematological malignancies, so hopefully may also be useful for treating colon cancer. — JDB

Sci. Signal. **7**, ra92 (2014).

IN OTHER JOURNALS

Edited by **Kristen Mueller**
and **Jesse Smith**



ATMOSPHERIC PHYSICS

A light-induced lightning rod

Lightning storms are fantastic examples of the power of nature, but also extremely damaging. They can destroy buildings, create power surges, are a major cause of forest fires, and make golfers abandon their games to seek shelter. Using light pulses fired from a powerful laser, Scheller *et al.* demonstrate the possibility of taming this wild force of nature. In a lab-based experiment, intense pulses of light vaporized a column of air and created a conducting filament of electrons that then acted as a lightning rod to channel the electrical discharge to ground. Scaling up this approach could enable the control of lightning strikes. — ISO

Optica **10**, 1364/OPTICA.1.000125 (2014).

RNA STRUCTURE

Unlocking the secrets of RNA in 3D

RNA can both store information in its linear sequence and take on critical structural and catalytic roles in the cell, such as during the translation of messenger RNA into proteins. These latter functions depend on the

complex higher-order structures RNA is able to form. Homan *et al.* now report a method to probe these intricate conformational states. They chemically modified exposed segments of three complex RNA structures. They then sequenced the RNA to map the locations of the multiple modifications in each individual linear RNA molecule. This allowed the

ALSO IN SCIENCE JOURNALS

Edited by Stella Hurtley

BIONANOTECHNOLOGY

Biological sensing using nanoparticles

Colloidal fluorescent and plasmonic nanoparticles yield intense responses to incident light, making them useful as sensors or probes for sensitive detection in solution. Howes *et al.* review the potential uses of nanoparticle biosensors in research and diagnostics. A range of methods allow for the chemical modification of the particle surfaces so that they can be tuned for specific analytes and give optical signals for a range of biological conditions of interest. Signals can be detected in complex media or in vivo making the particles of interest for both laboratory research and in clinical settings. — MSL

Science, this issue p. 53

STEM CELL SIGNALING

Controlling stem cells and their niches

Adult organs such as the intestines and skin continually renew themselves every few days or weeks. In several mammalian tissues, this renewal relies on Wnt signaling. Clevers *et al.* review this crucial role in stem cell self renewal. Wnt plays a pivotal role in tissue regeneration even in the earliest animals. Wnt proteins

function mainly as short-range signals between adjacent cells. The short-range, spatially-constrained nature of Wnt signals underpins mammalian stem cell niche architecture and tissue self-organization. — BAP

Science, this issue p. 54

NANOPHOTONICS

Controlling the flow of light with nanoparticles

Light propagating through optic fibers could provide the ultimate in information flow, but controlling the direction of flow is a key requirement. Petersen *et al.* show that the directional flow of light in a fiber can be controlled by placing a single gold nanoparticle on or near the surface of the fiber. By exploiting the chiral properties of light (the spin-orbit interaction), the authors demonstrate that the “handedness” or polarization state of the light hitting the particle determines in which direction the light flows in the fiber. — ISO

Science, this issue p. 67

PROTEOMICS

Mapping human drug targets in the cell

To understand both the beneficial and the side effects of a drug, one would need to know

its full binding profile to all cellular proteins. Savitski *et al.* take significant steps toward meeting this daunting challenge. They monitored the unfolding or “melting” of over 7000 human proteins and measured how small-molecule binding changes individual melting profiles. As a proof of principle, over 50 targets were identified for an inhibitor known to bind a broad spectrum of kinases. Two cancer drugs, vemurafib and Alectinib, are known to have a side effect of photosensitivity. The thermal profiling approach identified drug-protein interactions responsible for these side effects. — VV

Science, this issue p. 55

PROSTATE CANCER

Mutant protein in tumors hits the DEK

Cancer genome sequencing projects have uncovered a multitude of mutations in human tumors. Understanding whether and how these mutations contribute to tumor development and progression could ultimately lead to new therapies. Theurillat *et al.* studied the protein product of a gene that is recurrently mutated in prostate cancer. Normally this protein helps attach a biochemical tag to cellular proteins that marks

them for degradation. The new work shows that the tumor-associated mutant protein loses this tagging ability, which results in the stabilization of a handful of cellular proteins that would otherwise be degraded. One of the most intriguing of these proteins was DEK, which helps prostate cancer cells invade into surrounding tissue. — PAK

Science, this issue p. 85

CONSERVATION

Oil palm may become a threat to tropical forests

Palm oil is used in consumer products from margarine and ice cream to shampoo and detergents. Vast forests in East Asia have been cleared to create oil palm plantations. Higher-yielding oil palm varieties would allow more oil to be produced on the same amount of land. Could such varieties help to save further forests from destruction? In their Perspective, Carrasco *et al.* argue that the more likely outcome is further deforestation in the tropics as oil production shifts from temperate to tropical areas. This shift may result in land being spared in temperate areas. However, tropical forests are likely to come under further threat, particularly in Africa and South America. — JFU

Science, this issue p. 38

REVIEW

BIONANOTECHNOLOGY

Colloidal nanoparticles as advanced biological sensors

Philip D. Howes, Rona Chandrawati, Molly M. Stevens*

Colloidal nanoparticle biosensors have received intense scientific attention and offer promising applications in both research and medicine. We review the state of the art in nanoparticle development, surface chemistry, and biosensing mechanisms, discussing how a range of technologies are contributing toward commercial and clinical translation. Recent examples of success include the ultrasensitive detection of cancer biomarkers in human serum and in vivo sensing of methyl mercury. We identify five key materials challenges, including the development of robust mass-scale nanoparticle synthesis methods, and five broader challenges, including the use of simulations and bioinformatics-driven experimental approaches for predictive modeling of biosensor performance. The resultant generation of nanoparticle biosensors will form the basis of high-performance analytical assays, effective multiplexed intracellular sensors, and sophisticated in vivo probes.

Evolution has given rise to organisms of staggering complexity. Our now extensive knowledge of biological systems pales in comparison to the remaining mysteries. Unraveling these requires tools that probe the molecular machinery of life and provide detailed feedback on complex networks of subtle interactions. Such tools are cornerstones of biomedical research and practice, and improvements in these lead directly to a better understanding of fundamental biology, monitoring of health, and diagnosis of disease. Colloidal nanoparticle biosensors are a class of biological probe that will not only yield improved biological sensing but also provide a step change in our ability to probe the biomolecular realm. Nanoparticles can act as high-performance sensors because nanomaterials exhibit unique and useful behaviors not present in their bulk form: for example, bright tunable fluorescence from semiconductor nanoparticles and localized surface plasmon resonance (LSPR) phenomena in metallic nanoparticles. These particles exhibit intense responses to incident light (or other stimuli), and the ability to modulate this response by interaction with target analytes makes them excellent biosensor signal transducers. Outputs can be quantitative or qualitative depending on the functionality of the sensor; with both in vitro and in vivo applications. Nanoparticle biosensors can dynamically interact with and respond to their environment, which can be a considerable advantage compared to passive labeling techniques with nanoparticles, dyes, and stains. Another key advantage is that washing steps are often not required, as they are with passive labeling, making these biosensors suitable for

simple, rapid analytical quantification assays, detecting analytes in cells, and sensing and tracking analytes in vivo in real time.

Fluorescent quantum dots (QDs) and plasmonic gold nanoparticles (AuNPs) are commonly used in nanoparticle biosensors. QDs are fluorescent inorganic semiconductor nanoparticles, typically 2 to 10 nm in diameter, that possess high fluorescence brightness and photostability (1, 2). Their broad absorption and sharp emission spectra are tunable by particle size, allowing multiplexing where a mixed population of QDs can be excited with a single excitation source. Energy transfer between QDs and proximal donors or acceptors can be extremely efficient, and they can be functionalized with large numbers and varieties of functional molecules, factors that are vital for QD biosensors (3). Plasmonic nanoparticles exhibit unique optical properties due to LSPR (4). AuNPs receive particular attention because of their stability and ease of synthesis. Spatial confinement of surface plasmons in plasmonic nanoparticles yields a pronounced extinction peak in the visible spectrum, and intense surface fields polarize the local volume around the nanoparticles. External agents entering this volume (e.g., biomolecules, ions, dyes, other nanoparticles) interact with the field, leading to various effects, including LSPR peak shift, surface-enhanced Raman scattering (SERS), and quenching or enhancement of fluorophores. This yields an optical signal that can be modulated by the presence of an analyte, forming the basis of a biosensor (see Box 1 for typical nanoparticle biosensor architectures).

Analytical techniques such as enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), and fluorescence in situ hybridization (FISH) are the workhorses of biomolecular research and diagnostic laboratories. However, they are labor intensive, requiring various combinations of washing, heat cycling, and incubations.

Probing the molecular content and function of cells is currently reliant on organic fluorescent dyes; however, these offer poor photostability, broad absorbance and emission, and small Stokes shifts that make prolonged or multiplexed analysis very difficult. For in vivo sensing and imaging, near-infrared fluorescence can yield extremely high resolution real-time imaging and tracing of analytes but is limited by the poor spectral qualities of current dyes. All of these applications will benefit from robust and highly sensitive nanoparticle biosensors. The introduction process could follow two paths: Nanoparticle biosensors could form the basis of competing technologies to replace existing approaches: for example, an ultrasensitive nanoparticle biosensor assay to quantify proteins in physiological samples could replace ELISA; or, they could become advanced components that enhance the performance of existing technologies: for example, directly replacing the fluorescent dyes used in PCR, FISH, cellular fluorescence microscopy, and in vivo sensing.

Nanoparticle biosensors have achieved a high level of development, but there are still hurdles that must be overcome. The main challenges include reproducible synthesis of high-quality nanoparticles and maximizing performance in physiological conditions. Deploying these sensors in unfiltered and unpurified bodily fluids such as blood, urine, or saliva is challenging owing to the effects of interferent molecules, ionic concentrations, and changes in pH; therefore, great care must be taken to engineer sensors to withstand such conditions. Overcoming these challenges requires careful consideration of nanoparticle cores, surfaces, and biosensing mechanisms.

Nanoparticle cores

Nanoparticles for biosensing must be chemically robust to withstand complex conditions; show minimal perturbation of the probed system (e.g., low or no toxicity); and produce intense but switchable responses to incident light, yielding a strong signal change upon analyte interaction. For fluorescent biosensors, the optical properties of QDs are an excellent basis for signaling (5). However, the heavy-metal content of standard-composition QDs (typically CdSe and ZnS) means that toxicity might prohibit wide-scale use, particularly in clinical applications where disposal costs of toxic heavy metals could be prohibitive. Toxicological concerns have driven development of heavy metal-free alternatives with materials like silicon (6), carbon (7), ternary I-III-VI alloys of Cu, Sn, Zn, Ag, In, and S (8), and conjugated polymers (9, 10), and biosensing has been demonstrated with all of these. The challenge is to replace heavy metals while maintaining excellent properties, and as yet none of the alternatives have surpassed Cd-QDs in terms of optical quality and biofunctionalization. However, research activity is intense, and new formulations are constantly emerging. It is likely that some will eventually supersede Cd-QDs and have a substantial impact on the translation of nanoparticle biosensors.

Fluorescent nanoparticles absorb high-energy light and emit at a lower energy. Luminescent

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upconverting nanoparticles (UCNPs), a recently emerged class of nanoparticle, do the opposite, harvesting low-energy light by multiphoton absorption and upconverting to higher-energy emission. This property is excellent for biosensing, as UCNPs can operate at red-infrared wavelengths, where autofluorescence is avoided and greater penetration in biological matrices occurs (11). Current work is striving to improve the luminescence

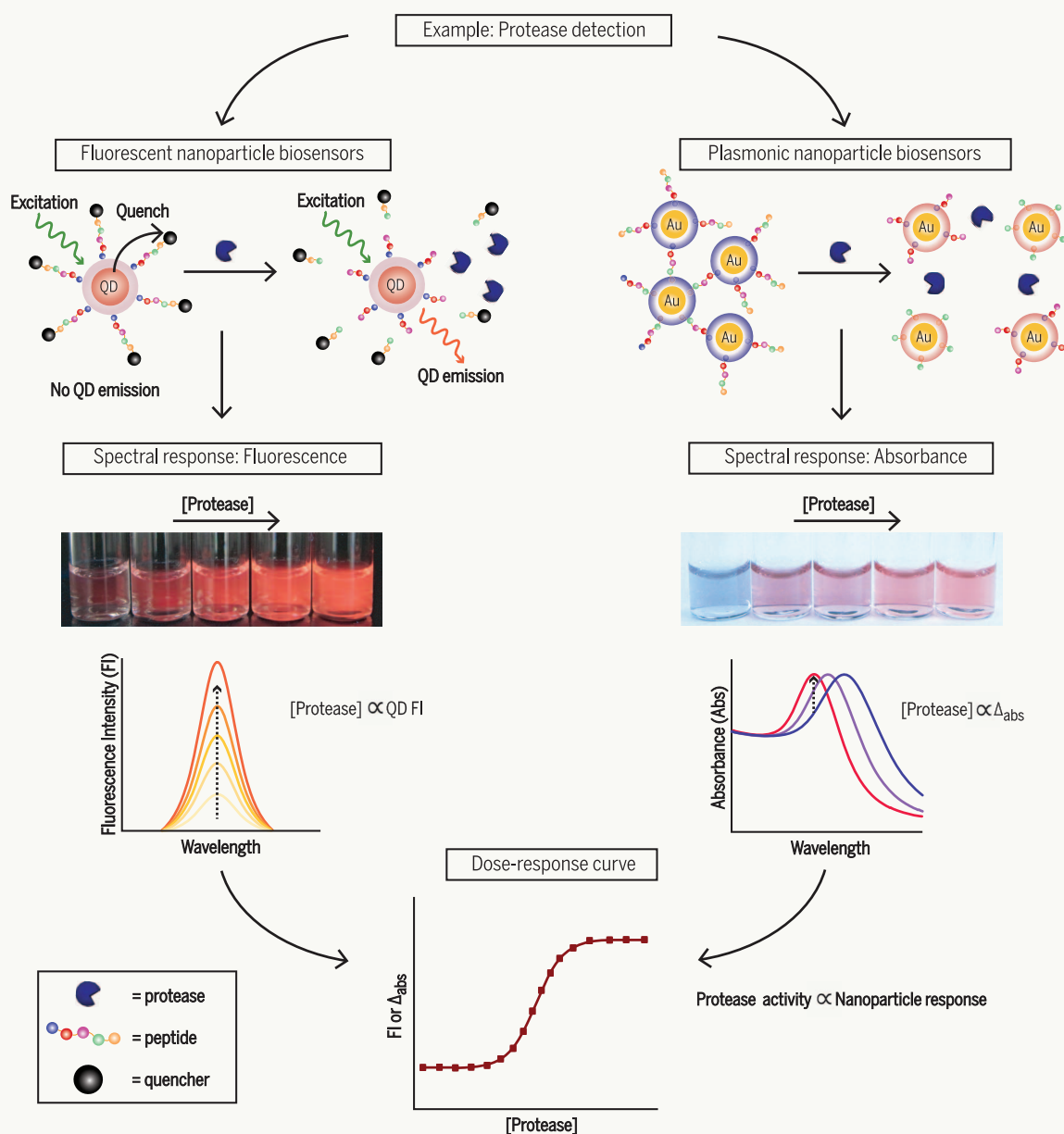
brightness and surface chemistry of these nanoparticles, and although application in biosensing is only a recent advance, it is becoming the next thrust of the field.

Unique phenomena emerge as the shape and size of noble-metal nanoparticles vary. Below ~2 nm, noble-metal nanoparticles (including Au, Ag, and Pt) exhibit fluorescence tunable across the ultraviolet, visible, and near-infrared (NIR) wavelengths

(12). Their biocompatibility, strong fluorescence, long emissive lifetimes, and excellent photostability make them attractive for nanoparticle biosensing (13). A major advantage of noble-metal nanoparticles over QDs is their small size, which is below the 5.5-nm renal clearance limit (14). Above 2 nm, noble-metal nanoparticles exhibit LSPR, which gives rise to superquenching, fluorescence enhancement, and LSPR field perturbation

Box 1. Nanoparticle biosensors.

Biosensors contain three general components: (i) a biologically derived and inspired recognition element to specifically interact with a target analyte; (ii) a signal transduction element to transform an analyte-receptor event into a signal; and (iii) a reader that interprets the signal and outputs a result. Nanomaterials such as quantum dots (QDs) and plasmonic gold nanoparticles (AuNPs) make excellent signal transducers, in which the signal derived from the material's unique optical properties is modulated by the presence of a target analyte. Here we illustrate detection of a protease enzyme that cleaves a linker peptide.



phenomena, which are all used for biosensing (15). The production of precisely engineered metal alloys or core-shell architectures allows fine tuning of plasmonic properties (16, 17). Common alloying elements include Cu, Pd, and Pt, and new synthesis routes are in active development (18). Furthermore, changing the shape of noble-metal nanoparticles shifts the LSPR peak position, allowing tuning for specific applications.

Beyond core composition, the way in which nanoparticles are synthesized also has a tremendous influence on their character. Nanoparticle properties are extremely sensitive to size, shape, crystal structure and associated defects, dopants, surface morphology and charge, and density of capping ligands. Although this sensitivity allows control and tuning of particle characteristics, it also makes syntheses very difficult to reproduce. Solution-phase synthesis, where precursors react in solutions containing coordinating ligands, yields high-quality nanocrystals with optimal physical and chemical characteristics and allows control over nanoparticle structure and morphology. Reaction parameters such as stirring rate, vessel morphology, and precursor injection position, which are often thought to be of minor importance in traditional synthetic chemistry, are critical in nanoparticle synthesis. Reproducible synthesis is therefore a major hurdle. Analyzing, understanding, and optimizing nanoparticle synthesis procedures is important both in the development of novel nanoparticle formulations and in moving toward robust nanoparticles for commercialization and advanced applications of nanoparticle biosensors. Complete control of reaction parameters and conditions can be achieved with automated robotic systems, such as the WANDA system at the Lawrence Berkeley National Laboratory (Fig. 1A) (19). Batch-to-batch variability can be avoided with continuous-flow systems, and incorporation of “in-line” analytical platforms (e.g., absorbance and fluorescence spectroscopy) that feed information back to reaction controllers allows real-time tuning and optimization of products, which is impossible with batch synthesis (20). Also noteworthy are microfluidic synthesis platforms, which have reduced reaction volumes with increased uniformity in the chemical and thermal environment and allow precise control over reaction conditions (Fig. 1B) (20, 21). Microfluidic systems will likely have a key role in optimization

and discovery of nanoparticle compositions and synthesis procedures. Given the extremely low reaction volumes, rapid throughput, and real-time tuning of product properties in continuous-flow formats, these systems can yield a large amount of information for relatively low inputs of reagents, time, and energy (22).

Nanoparticle synthesis methodologies have progressed substantially in the last two decades, but there is still much to learn about the exact nature of nanoparticle formation and growth. Examples include elucidating the importance of nonclassical (aggregation, coalescence) growth mechanisms in which particle-particle interactions play an important role (23, 24) and the effects of capping ligand concentration on growth trajectory (25–27). Unraveling these mysteries will influence development of sophisticated syntheses that yield reproducible results. A number of techniques have emerged to probe the processes involved in nanoparticle growth (Fig. 2). In situ x-ray irradiation from synchrotron sources can be used to follow various parameters during growth, including particle morphology, tomography, crystalline structure, size distribution, and particle assembly, and allows spectroscopic measurement of chemical and electronic configurations, all in real time (Fig. 2A) (28–31). Liquid cell transmission electron microscopy (TEM) allows real-time visualization of morphological, structural, and chemical changes of colloids (Fig. 2B) (23). A small volume of reaction solution is trapped between two electron-permeable membranes, such as graphene (32, 33), and energy delivered by the beam initiates particle nucleation. Techniques such as electron energy loss spectroscopy (EELS) and energy-dispersive spectroscopy (EDS) allow atomic-resolution imaging and chemical identification with liquid cells. Computer simulation is emerging as an excellent complement to these experimental techniques, allowing modeling and analysis of specific components and processes of nanoparticle nucleation and growth (Fig. 2C) (34). For example, density functional theory (DFT) has been used in combination with three-dimensional electron microscopy to study the growth of Ag shells on Au nanoparticles, revealing that growth on the (100) facets is preferred regardless of the morphology and crystallinity of the Au core (35). DFT has also been used to study the effect of alkanethiol capping ligands of different lengths

on the shape evolution of AuNPs during colloidal growth, showing that ligand concentration on particular faces drives variations in morphology (26). Furthermore, capping ligands themselves can be explicitly modeled with molecular dynamics (MD) simulations: for example, to reveal that cetyltrimethylammonium bromide (CTAB) forms a layer of distorted cylindrical micelles on Au nanorod surfaces and that channels among these micelles allow AuCl_2^- ions direct access to the surface during growth (27). These tools are changing our understanding of various processes, including particle nucleation, coalescence, shape control mechanisms, and surfactant effects (36).

Nanoparticle surfaces

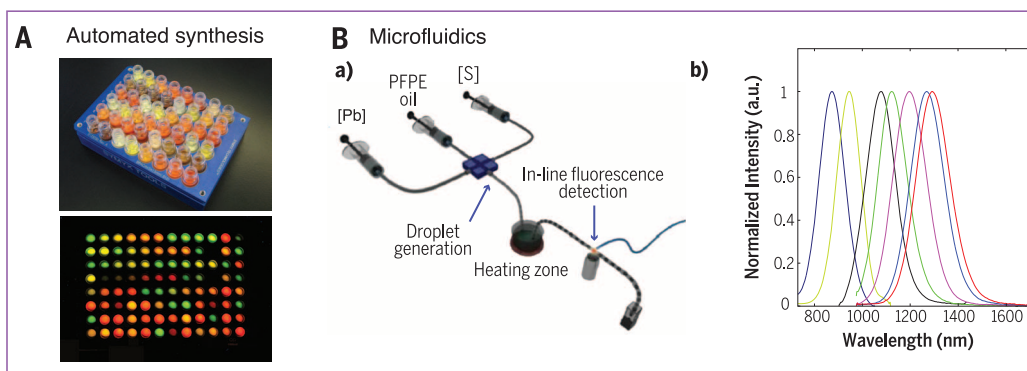
Nanoparticles require a surface covering to provide a barrier between the core and its environment. This is generally a layer of capping molecules that bind directly to the surface and ideally stops particles aggregating, disperses them in water at a range of pH values, resists nonspecific adsorption of surrounding molecules, and provides a conjugation point for functional biomolecules (Fig. 3). The interface between a nanoparticle core and a biological environment is a key area and must be engineered carefully to optimize nanoparticle biosensor performance (37).

The many biomolecules (enzymes, lipids, etc.) and ions in physiological fluids such as blood, urine, and saliva provide a hostile environment for nanoparticles; therefore, capping ligands must provide effective protection. For nanoparticle biosensors, capping layer thickness is key. The biosensing mechanisms that modulate nanoparticle signals in response to analyte interaction are usually governed by distance-dependent interactions across the capping layer, where thinner capping layers result in stronger signal modulation. These interactions include resonance energy transfer (RET), where energy is transferred across space (typically up to 10 nm) by nonradiative dipole-dipole coupling, and LSPR field perturbation, where molecules entering the LSPR field alter the refractive index of the sensing volume surrounding a plasmonic nanoparticle (typically up to 30 nm from the surface). This puts a constraint on the capping layer; it must be both compact and highly protective. Thinner capping layers are generally less protective against particle aggregation, nonspecific adsorption, and surface degradation; hence,

Fig. 1. Nanoparticle synthesis.

(A) CdSe and CdTe QDs synthesized by WANDA, allowing reproducible synthesis and high-throughput optimization of nanoparticles (19).

(B) (a) A droplet-based microfluidic system for precision QD synthesis (PFPE, perfluoropolyether) with in-line fluorescence detection. (b) Spectral tuning of PbS QDs with automated control over temperature (95° to 155°C, increasing in 10°C increments), holding reaction time and Pb:S:surfactant ratios constant (20) [Adapted with permission from (19, 20)]



there is an inherent conflict between decreasing capping thickness and increasing protection.

The most successful surface-capping approaches to date have made use of custom molecules with modular combinations of low-molecular weight components that convey a range of beneficial properties to the nanoparticle. A notable breakthrough is that of modular zwitterionic ligands that yield nanoparticles with exceptional stability and functionality (38–41). Through electrostatic interactions and hydrogen bonding, zwitterionic ligands can bind large numbers of water molecules that act to restrict nonspecific interactions with surrounding biomolecules (42, 43). This is important, as nanoparticles show a propensity to adsorb proteins and other biomolecules, creating a “corona” of material that, if too thick or too strongly bound, will block access of analytes to surface-bound biosensing mechanisms. The zwitterionic groups (e.g., sulfobetaines, carboxybetaines) contain a diversity of charges with net zero charge, which allows stability over extended pH and ionic concentration ranges, an important attribute given the diverse characteristics of different physiological fluids. Ligands can be designed with multiple surface-binding groups (e.g., thiols, which form dative covalent bonds with many metals) to ensure strong and lasting binding: for

example, bidentate dihydrolipoic acid (DHLLA)-based (44) and tridentate tris(mercaptomethyl) ligands (45). Short ethylene glycol segments can also be included to control nonspecific adsorption (46). Zwitterionic ligands yield exceptionally stable nanoparticles and are the state of the art for compactness and fouling resistance, particularly when combined with novel phase transfer routes like photoligation (47).

Polymeric capping layers offer several advantages, including many surface-binding groups per molecule and increased control over functional group number and position, but historically it was difficult to obtain compact layers owing to their relative bulk. However, improved control over polymerization and surface coordination has yielded excellent polymer capping agents (48). Examples include hydrophilic polymers grafted with short thiolated alkyl chains (49) and reversible addition fragmentation chain transfer (RAFT)-mediated polymerization of mixed-functionality monomers (50). Given that thiol oxidation is problematic and seemingly unavoidable, research is starting to avoid their use; thus, inclusion of groups such as imidazoles as coordinating moieties is noteworthy (51).

The exact nature of the nanoparticle–environment interactions is yet to be elucidated, and there are

several phenomena that have a notable effect upon the functionality of nanoparticle biosensors (52). Charged nanoparticles attract large numbers of ions that alter the local environment, creating ionic and pH gradients. These can affect the biosensing mechanisms (e.g., protein–antibody binding or DNA–DNA hybridization) and the conformation of component biomolecules. Nanoparticles are similar in size and shape to many proteins, and their interactions are mediated by the same forces, such as van der Waals interactions, dipolar attractions, electrostatic attraction and repulsion, and hydrogen bonding. They can therefore undergo dynamic interactions with each other that can alter properties of both nanoparticle (e.g., inducing phase transformations, restructuring, and dissolving the nanoparticle surface) and protein (e.g., denaturation, altering enzyme activity). Precise understanding of these emerging phenomena is important to inform the design and development of nanoparticle biosensors, and such studies are currently a key part of nanoscience (53).

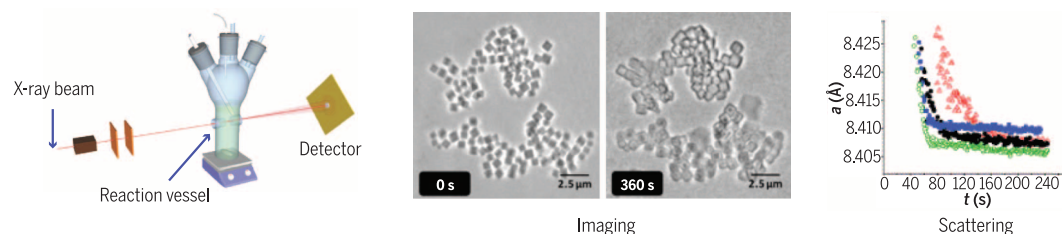
Biosensing mechanisms

Modulation of nanoparticle signals by interaction with target analytes is governed by the architecture of surface-bound biosensing mechanisms. The key elements are (i) the way constituent

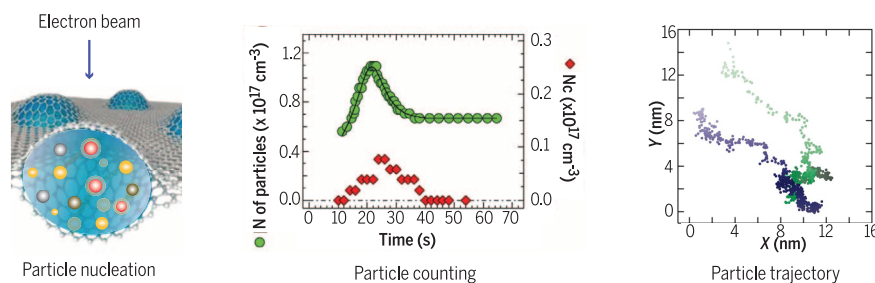
Fig. 2. Analysis of nanoparticle nucleation and growth. (A) In situ

synchrotron x-ray techniques (left) allow real-time probing of colloidal nanoparticle growth: for example, x-ray imaging [growth of CuS shells on CuO cores over 360 s; scale bar, 2.5 μm (28)] (middle) and scattering [tracing the evolution of crystal structure by measuring the unit cell dimensions of magnetite nanoparticles reducing during growth (30)] (right). (B) Liquid cell TEM allows direct visualization of growing nanoparticles (left), following, for example, the number of particles (N , left axis) and number of coalescence events (N_c , right axis) versus time (23) (middle), and trajectory before coalescence (right) (32). (C) DFT studies of alkanethiols on AuNPs (left) reveal the most energetically favorable binding sites (middle, shown in red) and how ligand length can directly influence particle morphology (right) (26). [Adapted with permission from (23, 26, 28, 30–33)]

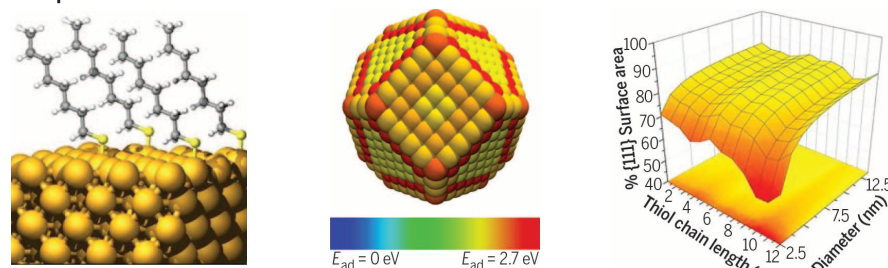
A In situ x-ray irradiation



B Liquid cell transmission electron microscopy



C Computer simulation



biomolecules are conjugated to the nanoparticle, (ii) the signaling modes employed, and (iii) the analyte-receptor mechanism.

Bioconjugation is used for constructing biomolecular sensing mechanisms (Fig. 3B) (3). Biomolecular recognition elements are immobilized on particle surfaces, and their interaction with analytes yields a physical or chemical response that modulates the particle-derived signal (Fig. 4, A to G). Traditional covalent approaches often require multiple washing and purification steps, hydrophobic reaction intermediates, and multiple reagent additions that often destabilize colloidal nanoparticles. Unintended cross-linking between nanoparticles, and between biomolecules, is also a common problem, and such techniques allow little control over the number of conjugated species.

Ideal bioconjugation procedures should be simple, high yield, and nondamaging to nanoparticles and preferably should require no intermediate reagents. The binding of biotinylated biomolecules to streptavidin-coated nanoparticles achieves all of these characteristics; however, the relative bulk of streptavidin (52.8 kD, ~6 nm) severely restricts RET efficiency. Oligohistidines are excellent alternative bioconjugation vectors, as they can bind directly to particle surfaces, sitting between surface ligands. They have been used on both QDs and AuNPs (54). A short sequence of histidine amino acids can be included in a recombinant protein (54), in a peptide sequence (55), or as part of an oligonucleotide construct (56), and its high affinity for metal nanoparticle surfaces allows attachment with

excellent control over number and orientation of attached species. The many emerging highly site- or sequence-selective conjugation techniques, such as click reactions (57, 58), strain-promoted cycloadditions (59), and enzyme-mediated ligations (60, 61), are excellent prospects for nanoparticle bioconjugation, as they offer rapid, efficient, and strong binding. Synthetic biomolecules, such as peptides and DNA oligomers, can easily be functionalized with the required groups. Proteins can have appropriate functional groups inserted into their peptide sequence by the recombinant inclusion of non-natural amino acids. This yields proteins that can be conjugated to nanoparticles with high control over both number and orientation of attached species (62). Such precise control over position and selective reactivity of conjugating groups is an important step toward a sophisticated “plug-and-play” approach to nanoparticle bioconjugation (3).

Signaling modes are key for nanoparticle biosensors. Fluorescence-based tools are used extensively in biological sciences—for example, in analyte quantification assays and cellular imaging—and they offer high resolution and excellent sensitivity. Sensing mechanisms of fluorescent nanoparticle biosensors generally involve charge or energy transfer to modulate fluorescence intensity, lifetime, or spectral profile. Charge transfer acts by perturbing excited states in the particle core to either enhance or quench emission. RET processes include Förster resonance energy transfer (FRET) and bioluminescence or chemiluminescence (BRET and CRET) and can involve a variety of materials, including organic dyes, lanthanide complexes, fluorescent proteins, graphene, chemi- and bioluminescent enzymes, and inorganic materials like AuNPs, which may function as acceptors or donors and can be arranged in relays (63). The use of long-lifetime luminescent nanoparticles and RET agents allows for time-gated detection, which will be an important area of development for commercial nanoparticle biosensors (64). Minimizing donor-acceptor separation is paramount, and nanoparticle biosensing components must be carefully chosen and constructed to achieve maximum sensitivity.

Plasmonic nanoparticles act as biosensor signal transducers in three main ways. First, the LSPR peak can shift in response to the action of an analyte, usually matter entering or exiting the LSPR field. Such matter may be analytes binding directly to the particle (65), plasmonic nanoparticles aggregating or disaggregating (66), or selective deposition of a metallic layer on the nanoparticle surface (67). Second, enhancement of Raman scattering intensity due to the binding of secondary agents to plasmonic nanoparticles allows sensitive detection: for example, by binding analytes directly to nanoparticles (direct intrinsic SERS) (68), modulation of SERS due to perturbation of a receptor (indirect intrinsic SERS) (69), or selectively adding or removing Raman-active dyes (extrinsic SERS) (70). Third, plasmonic nanoparticles can enhance or quench nearby (<10 nm) fluorophores through interactions of their excited electrons with the LSPR field (71),

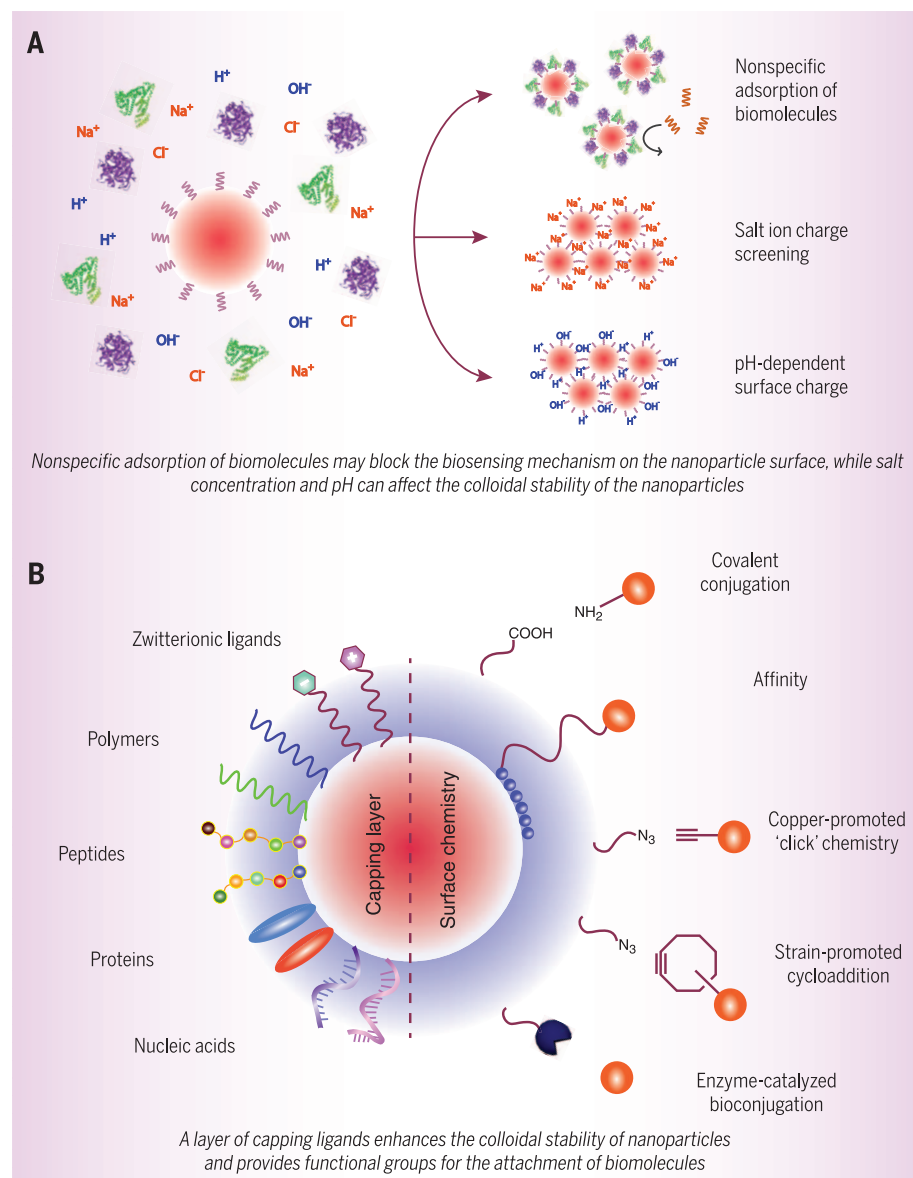


Fig. 3. Nanoparticle stability and surface chemistry. (A) The nature of nanoparticle capping ligands is fundamental in determining the functionality of nanoparticle biosensors in complex solutions. (B) Many capping ligands have been used, and their functional groups are key in the construction of the biosensing mechanisms.

which allows for combining advanced plasmonic and fluorescent nanoparticle constructs.

Analyte–receptor interaction events activate the biosensing mechanisms that modulate nanoparticle properties. Examples include the following:

1) Enzyme–substrate: Enzymes are essential in maintaining physiological homeostasis, and aberrant expression or activities act as biomarkers for various diseases. Systems to measure enzyme activity and abundance are vital in both research and clinical practice. Enzyme substrates, such as proteins and peptides, can act as active elements in biosensing mechanisms (55). For example, a RET-active agent tethered to a fluorescent nanoparticle by a short protease-selective peptide sequence can be removed by protease cleavage, modulating nanoparticle fluorescence (Fig. 4A) (72, 73). Enzymes can also alter the aggregation state of plasmonic nanoparticles: for example, by protease cleavage of peptide-bound aggregates of AuNPs (74), kinase-induced aggrega-

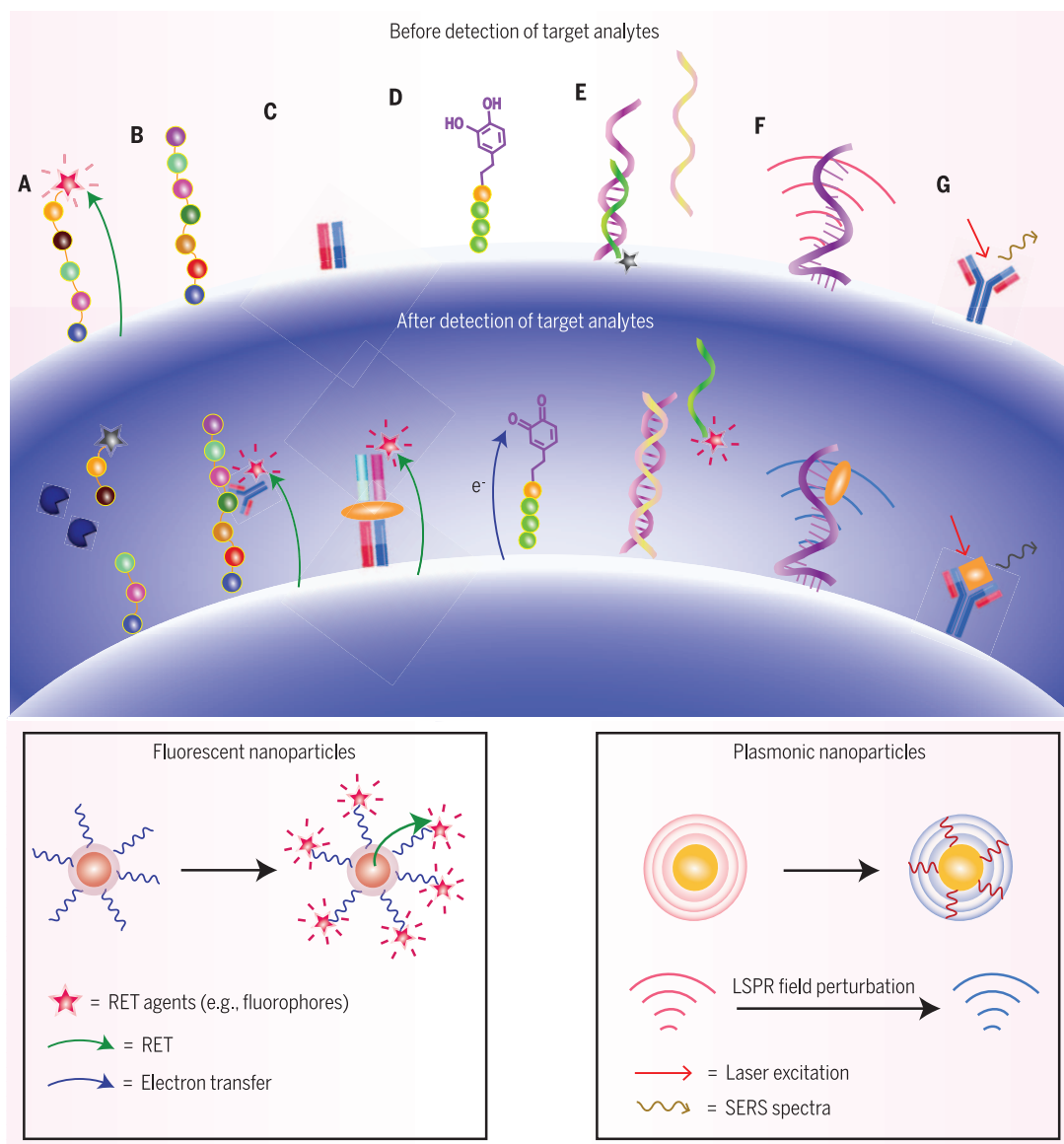
tion of peptide/antibody-coated AuNPs (66), or transglutaminase-induced covalent cross-linking of peptide-coated AuNPs via the formation of iso-peptide bonds (75).

2) Antigen–antibody: Antibodies are used in protein biosensors for their high specificity and form the basis of ELISA. The advent of controlled antibody fragmentation and single-domain antibodies is yielding compact immunosandwich complexes that allow for low limits of detection (LOD) in nanoparticle biosensing applications (76). Nanoparticle FRET biosensors have been constructed with such fragments (Fig. 4C) (77). For plasmonic nanoparticles, proteins binding to antibody-functionalized surfaces can induce a measurable LSPR (65), or a secondary antibody can be used to introduce an additional agent for signal amplification (67). Furthermore, studying the SERS spectra of receptor or bridging molecules, such as antibodies, upon binding an analyte allows plasmonic nanoparticles to act as effective label-free biosensors (Fig. 4G) (69).

3) Nucleic acid interactions: Nucleic acids can perform complex structural and mechanistic roles in engineered systems, and they are excellent for constructing biosensing mechanisms. For nucleic acid targets, molecular beacons are common and have been used in nanoparticle-based configurations (78). Nucleic acid displacement can selectively remove donors or acceptors from a double-stranded complex on a nanoparticle surface, which is the basis of nanoflares (Fig. 4E) (71). Aptamers are nucleic acid sequences that can selectively bind various analytes, including proteins. They have several advantages over antibodies (e.g., compactness) and make excellent sensing elements (Fig. 4F) (79).

4) Redox reactions: The intrinsic redox properties of certain biomolecules allow nanoparticle biosensors to measure pH. This is useful for intracellular biosensing: for example, where pH can modulate many cellular events. An example is dopamine, which can directly react with O_2 to convert to quinone under basic pH. As quinone

Fig. 4. Surface-bound biomolecular sensing constructs. Example biosensing mechanisms based on (A, B, and C) resonance energy transfer, (D) charge transfer, (E) fluorescence superquenching, (F) LSPR field perturbation, and (G) SERS spectral shift. Analyte–biomolecule interactions are (A) protease–peptide (73), (B) transferase–peptide (55), (C) protein–antibody fragment (77), (D) dopamine response to pH change (80), (E) nucleic acid hybridization (nanoflare) (71), (F) protein–aptamer (79), and (G) protein–antibody (65).



is an electron acceptor, it can quench QDs by charge transfer (Fig. 4D) (80).

Moving toward applications

Fundamental nonclinical research is likely to be one of the first areas where nanoparticle biosensors will have a substantial impact given the fewer restrictions and regulatory hurdles compared to clinical application. We can envision nanoparticle biosensor-based assays as replacements for many standard research tools. For example, ELISAs allow quantification of proteins in complex media but are slow, with many incubation and washing steps. Instead, nanoparticle biosensors added to a sample could readily output a quantifiable optical signal. This has been demonstrated with QDs, where prostate-specific antigen (PSA) was detected in serum by using antibody fragments, long fluorescence-lifetime lanthanide dyes, and time-gated fluorescence detection (77). PCR is robust and reliable for DNA or RNA detection but requires sophisticated equipment and lengthy protocols. Instead, a one-pot nanoparticle biosensor assay could detect and quantify targets in complex mixtures, which has been demonstrated with superquenching AuNPs (81). Such assays could be kit-based, and users would generally be well skilled; therefore, these types of application are easy to envisage and would fit in easily alongside other research tools and methodologies.

Understanding of biology, and ultimately disease, diagnosis, and cure, is reliant on understanding what transpires in and around cells. This requires sensitive, reliable, reproducible, and highly stable tools. The application of nanoparticle biosensors as active cellular probes is a highly promising area, and they are beginning to achieve success (Fig. 5A) (78, 82). Getting nanoparticles into intracellular regions of interest is a major challenge. Cellular cytosol, the intracellular fluid in which substructures (organelles) are suspended, is a major target for intracellular sensing. Cells most commonly take up matter by endocytosis, enveloping it in endosomes and not exposing it to the cytosol. To reach the cytosol, nanoparticles must break out of endosomes or find alternative internalization pathways. Standard delivery methods include microinjection and electroporation, but these can affect cell viability. Cell-penetrating peptides (CPPs) offer a nonmechanical “lock and key” type of entry (83). Peptides containing His-tag QD-binding motifs and CPP segments have been used to achieve endosomal escape and cytosol entry (84), and intracellular detection of Ca^{2+} ions has been demonstrated with QD-CPP biosensing constructs (85).

Analytical tools developed for research can translate into *in vitro* clinical diagnostics, though complications regarding cost, regulation, and rapidity make this very challenging (86). A particular area of interest is in nanoparticle biosensors for point-

of-care diagnostics—for example, in microfluidic “lab-on-a-chip” devices (87) or merging with technologies such as smartphones (Fig. 5B) (88) and Google Glass (89). For such applications, nanoparticle biosensors must be stable enough to withstand long-term storage and fluctuations in environmental conditions. These are challenges for all reagents but will be particularly so for nanomaterials given their complex chemistry. These applications are a mid-to-long-term goal, and advances in the synthesis and surface engineering of nanoparticles are imperative.

Using sophisticated “turn-on” *in vivo* biosensors that could signal upon binding specific targets (e.g., cancer cells) would be a far-reaching development, and the high absorptivity and strong fluorescence of many nanoparticles make them highly suitable. *In vivo* translation introduces many complexities regarding regulation, short- and long-term toxicity, undesired rapid capture by the immune system, routes of degradation or escape, and environmental impacts of cleared nanoparticles, and nanoparticle biosensors must be carefully designed to circumvent these issues. There are also physical limitations, such as autofluorescence and low tissue penetration by light due to high scattering and absorbance, which make probing biological structures and tissues with light problematic. However, these impediments can be sidestepped with the use of certain materials and techniques. By working in the

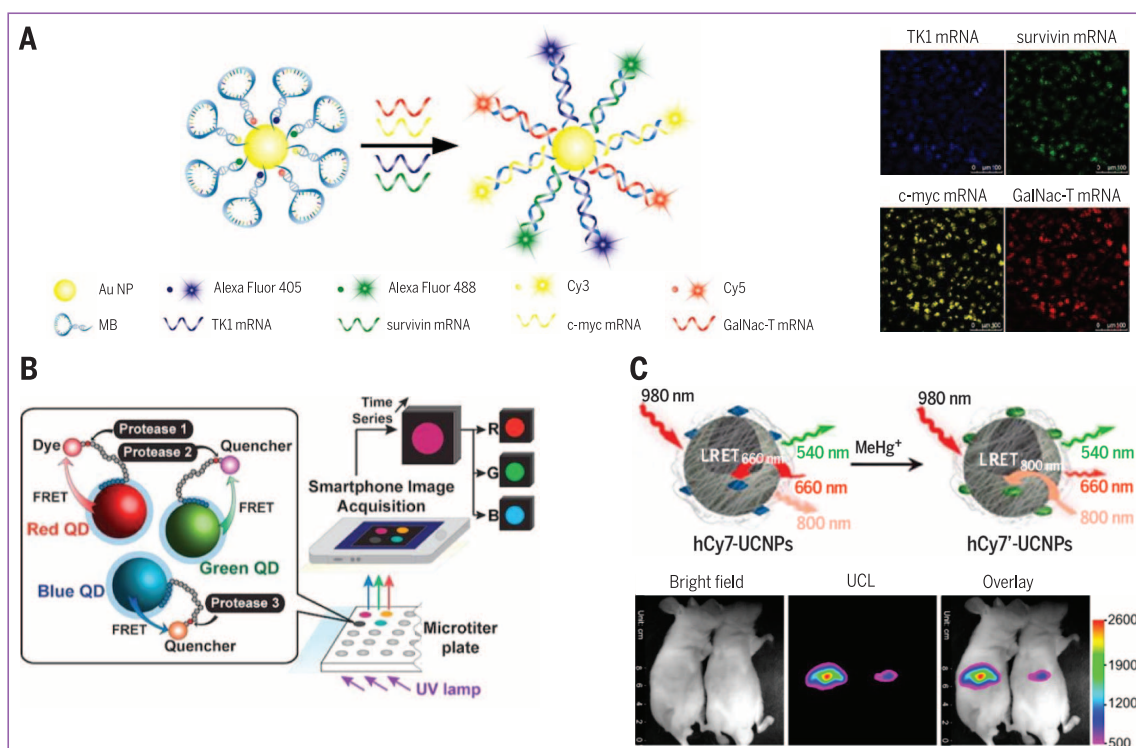


Fig. 5. Applications of nanoparticle biosensors. (A) Multiplexed intracellular sensing of four mRNAs in MCF-7 human breast cancer cells using AuNPs with a dense shell of multicolored molecular beacons. These show high nuclease resistance and biocompatibility (scale bars, 100 μm) (78). (B) Multiplexed sensing of protease enzymes using red-, green-, and blue-emitting QDs, where proteases cleave surface-bound dye-labeled peptides. Fluorescence is monitored

using the red, green, and blue channels from a smartphone camera (88). (C) *In vivo* sensing using rare-earth UCNPs with surface-bound NIR hCy7 dyes. Upconversion luminescence (UCL) imaging (excitation 980 nm, emission 800 nm) shows MeHg^+ accumulation in the livers of live mice due to quenching of UCL by MeHg^+ (right mouse) versus a control (left mouse) (90). [Adapted with permission from (78, 88, 90)]

near-infrared energy range, where scattering and absorbance of tissue are at a minimum, tissue penetration can be maximized. This can be done with UCNPs, which can undergo excitation and emission in the red-infrared bioimaging window (Fig. 5C) (11, 90). Furthermore, by using long-lifetime fluorophores, such as lanthanide dyes and time-gated fluorescence spectroscopy (97), it is possible to discard the initial short-lifetime autofluorescence burst and detect only the long-lifetime fluorescence signal that is reporting on the target. Another exciting prospect is in vivo photoacoustic sensing, which combines NIR excitation with ultrasonic detection based on the photoacoustic effect and yields higher spatial resolution and deeper tissue penetration than fluorescence techniques. For example, low-band-gap semiconducting polymer nanospheres (SPNs) with strong NIR absorbance have been used for in vivo sensing of reactive oxygen species (ROS) in live mice. Immobilization of a ROS-responsive cyanine dye derivative on the particle surface, and comparison of the photoacoustic response of the SPN at 700 nm and the dye at 735 nm, yielded a ratiometric response (10). Looking toward application of these techniques, clinical in vivo sensing still remains a long-term goal, but solid foundations have been built for this field.

It is useful here to reference a nanomaterial that has had great success in many applications. Spherical nucleic acids (SNAs) are nanoparticles, commonly AuNPs, with a dense DNA “shell” that is simultaneously a highly protective capping layer and an active biosensing construct (92). In the original work (93), a target DNA sequence induced AuNP aggregation, inducing an LSPR shift. This was developed into a microarray scanometric assay (94), which evolved into the Verigene System that can detect analytes for many different diagnostic ends, including clinical microbiology, as well as human genetic and pharmacogenetic testing (95). With a LOD of 100 aM for DNA targets, target amplification is not required as it would be for PCR-based methods, and the system has also been adapted to detect proteins (96) and microRNAs (miRNAs) (97). SNAs also allow intracellular biosensing and can enter cells in considerable numbers without transfection agents (98). Hybridizing dye-functionalized nucleic acid strands (nanoflares) to the shell turns the dyes “off” as a result of superquenching by the AuNP core. A toehold region in the probe allows a target nucleic acid to hybridize and remove the flare, whereupon the dye turns “on.” These are effective for measuring intracellular mRNA levels (77).

A perspective on the future of nanoparticle biosensors

Technology transfer is a great challenge for all applied research. Commercialization of biosensors in general has been slow, with glucose monitoring and pregnancy testing remaining the only large-scale consumer markets. Since the advent of functional bionanomaterials in the 1990s, there has been much excitement that combining these with advanced biosensing strategies could revo-

lutionize many research and clinical practices. While the abundance of proof-of-principle studies proves that nanoparticle biosensors can deliver, we still await effective technology transfer. Such transfer is absolutely vital for nanoparticle biosensors to achieve widespread use and impact.

We can identify five key areas on the materials front that will substantially influence the short-term development and translation of nanoparticle biosensors: (i) Nanoparticle cores with minimal toxicity and optimal optical properties are imperative for wide-scale applications. New candidates are constantly emerging and evolving, and the likes of heavy metal-free QD alloys, carbon dots, and metal nanoclusters may supersede Cd-QDs. (ii) Robust synthesis of whole nanoparticle biosensors is required for the manufacture of standardized products. Systems with precise control of all reaction parameters for multistep procedures are required: for example, continuous-flow reactors with built-in analytical and feedback mechanisms, and microfluidic systems for optimization and discovery. (iii) Compact capping agents that yield highly stable and resistant nanoparticles are essential: for example, modular small-molecule or polymer ligands including units such as zwitterionic groups, ethylene glycol segments, and imidazole groups, enhancing colloidal stability, nonspecific adsorption resistance, and surface binding. (iv) Bioconjugation methods are needed for fine control of number and orientation of surface-conjugated biomolecules. Ongoing transfer of ideas from highly selective and efficient protein-labeling chemistries that do not perturb structure or function will have a massive impact: for example, enzyme-mediated ligation and recombinant proteins with non-natural amino acids. (v) Robust RET agents are required that are more effective and stable than organic dyes, such as graphene and AuNPs acting as quenchers, or long-lifetime agents like lanthanides that allow time-gating (63).

Beyond materials development work, we identify five areas that we believe will be at the forefront of nanoparticle biosensor development:

1) Computer simulations to aid development and predict performance: Simulation will become a vital tool for understanding phenomena and optimizing performance in all areas of nanoparticle biosensor development (e.g., structure-property, surface-ligand-environment, and target-probe relationships). Simulation currently provides validation of experiment, but the increasing sophistication of algorithms and modern supercomputers will give these techniques predictive capabilities and great potential for success within the next decade. An example application is analyzing the structure of the metal-sulfur interface of thiolate-protected particles, which is being studied using DFT modeling in combination with scanning tunneling microscopy, low-energy electron diffraction, and surface-sensitive x-ray spectroscopic techniques (99). Such studies have revealed, for example, the importance of the complex RS-AU-SR in forming continuous “polymeric” structural features on the surface of AuNPs. Another area of importance will be elucidating the complex

phenomena that occur at the nano-bio interface (100): for example, understanding the specific and nonspecific interactions between proteins and ligand-capped nanoparticles, where MD simulations have already revealed how amphiphilic residues (e.g., arginine, tyrosine, tryptophan) play a key role in protein-particle interactions for mixed-monolayer-protected metal nanoparticles (101).

2) Bioinformatics approaches for predictive modeling: Identification of structure-activity relationships (SARs) helps predict biological activity from structure. Considering the complexity of biological environments, the vast array of engineered nanomaterials, and the emergent phenomena at the nano-bio interface, approaches that can handle vast amounts of experimental data are needed to create SARs outputs for nanoparticle-biomolecule constructs. Bioinformatics approaches will prove vital for this purpose, and recent work has demonstrated the power of turning systematic combinatorial experimental data into quantitative models with predictive power (102). Here, the interaction of 105 types of unique serum protein-coated AuNPs with human lung epithelial carcinoma cells was characterized and used to create a multivariate model that uses the protein corona fingerprint to accurately predict cell association (nanoparticle internalization and cell membrane adhesion). Such models will direct nanoparticle biosensor design to maximize specific and minimize nonspecific interactions to optimize performance in physiological fluids, and we expect to see work in this area increase rapidly.

3) Merging with developments in molecular diagnostics: There is intense research to identify panomic (genomic, proteomic, etc.) molecular markers of disease for screening, diagnosis, prognosis, and staging. The trend is toward identifying biomarker “panels” from large-cohort data sets to increase sensitivity and specificity versus single biomarkers. For example, a recent study revealed a panel of 10 lipids from peripheral blood that predicted, with >90% accuracy, phenocconversion to amnesic mild cognitive impairment or Alzheimer’s disease within 2 to 3 years (103). To use such panels for clinical diagnostics, or even rapid point-of-care testing, technologies that can simultaneously detect multiple analytes with ultralow LOD are vital. Ultrasensitive nanoparticle biosensor assays using the multiplexing capabilities of QDs (88), or fluorescent barcode nanoparticles for high-density multiplexing (104), are ideal for such application, and merging these fields will yield high-impact technologies and clinical approaches in the medium-to-long term. Furthermore, discovery of new types of biomarkers can feed back into biosensor development with the integration of the newly discovered target-analyte interactions into colloidal particle systems.

4) Integration of nanoparticle biosensors into assays and devices: The question of how nanoparticle biosensors fit into larger systems that go from raw sample to result is of paramount importance and the focus of much current research activity. An example is combining nanoparticle

biosensors with advanced target amplification protocols for the detection of nucleic acids, which can further decrease LOD. For example, a QD biosensor has been incorporated into an exponential amplification reaction to achieve a 0.1-aM LOD for miRNA (105). The U.S. Food and Drug Administration–cleared Alere i system (106), which provides detection and differentiation of influenza A and B in 15 min from a nasal swab, has demonstrated that isothermal amplification and fluorescence-based detection are viable in point-of-care technologies. We anticipate that integrating sophisticated nanoparticle biosensors into such systems will yield extremely high-performance technologies. Furthermore, given that nucleic acid targets can be easily amplified whereas other targets cannot (e.g., proteins, metal ions), it is likely that nanoparticle biosensors will have a more rapid impact in genomics than in other fields.

5) Application as intracellular sensors: We anticipate that this will be the first area of high-impact application of nanoparticle biosensors. We expect to see some high-profile examples appearing in the next few years, followed by extensive usage and ground-breaking work when nanoparticle biosensors break through as commercially available tools. A possible breakthrough application could be in probing cell-signaling networks, which are of critical importance in cancer. The healthy cell to cancer cell transformation involves many changes in behavior—for example, in proliferation, motility and survival—which are underpinned by complex alterations in cellular signaling. Understanding such pathways is important for both fundamental understanding of cancer progression and identifying specific drug targets. Conducting these studies with traditional techniques is laborious and slow. Instead, multiplexed “turn on” nanoparticle biosensors could allow extended monitoring of signaling events in live cells. Multiple branches or points in a signal cascade could be monitored simultaneously, with highly specific reporting on events such as phosphorylation or dimerization, to follow the flow of information and elucidate the nature of the cascade. Furthermore, such sensors could form the basis of next-generation theranostics, providing site-specific treatment with feedback mechanisms: for example, monitoring drug effects in real time and informing adjustment of dose and rate. The opportunities in this area represent probably the greatest potential for successful application of nanoparticle biosensors in biomedicine.

Nanoparticle biosensor research stands at a critical juncture, with a vast amount of excellent research that forms a solid foundation for future work. The field must push on to take its technologies from being lab curiosities to achieving large-scale application and impact, and we are now very well equipped with the tools necessary to do this.

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REVIEW

STEM CELL SIGNALING

An integral program for tissue renewal and regeneration: Wnt signaling and stem cell control

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Stem cells fuel tissue development, renewal, and regeneration, and these activities are controlled by the local stem cell microenvironment, the “niche.” Wnt signals emanating from the niche can act as self-renewal factors for stem cells in multiple mammalian tissues. Wnt proteins are lipid-modified, which constrains them to act as short-range cellular signals. The locality of Wnt signaling dictates that stem cells exiting the Wnt signaling domain differentiate, spatially delimiting the niche in certain tissues. In some instances, stem cells may act as or generate their own niche, enabling the self-organization of patterned tissues. In this Review, we discuss the various ways by which Wnt operates in stem cell control and, in doing so, identify an integral program for tissue renewal and regeneration.

In a 1956 review entitled “Renewal of Cell Populations,” Leblond and Walker noted that multiple adult tissues, including the skin and intestines, accommodate numerous mitotic divisions but seemingly do not undergo a commensurate expansion in tissue size (1). The authors presciently concluded that “the cells of the tissue are said to undergo renewal” (1). Such tissues are perpetually being “recycled,” with cells being extruded or lost and continually being replaced by newly born cells.

It is now evident that stem cells are required for continuous tissue maintenance within diverse organs. Cellular losses within these tissues (owing to either natural cellular attrition or injury) are persistently replenished by stem cells, which we define as cells that sustain continued tissue formation by generating tissue progeny while renewing themselves through division. Stem cell activity is often externally dictated by the microenvironment (the niche) so that stem cell output is precisely shaped to meet homeostatic needs or regenerative demands.

This Review details how a class of developmental signals, known as Wnt signals, control stem cell operation and are crucial for the continued renewal of multiple mammalian tissues. Such a role was presaged by a pivotal role for Wnt in the development and regeneration of the earliest animals. Although a number of signals control stem cell activity, Wnts are somewhat idiosyncratic in that they primarily seem to act as short-range cellular signals between adjacent cells. This

mode of spatially constrained signaling might bear developmental and regenerative importance, communicating a directive to nearby cells without influencing a broad domain.

Signaling by lipid-modified short-range Wnt factors

A tenet of the stem cell niche model is the short range at which signals act, maintaining a limited number of stem cells near the niche. By their very nature, Wnt proteins fit the bill.

Wnts are secreted signaling proteins that by virtue of their biochemical properties, seem principally to operate over short distances. All Wnt proteins harbor a covalent lipid modification: a palmitate, appended by the palmitoyltransferase Porcupine (Fig. 1A). This lipid group renders the Wnt protein hydrophobic and tethers it to cell membranes or its cognate receptors. The transmembrane protein Wntless (Wls) exclusively binds only lipidated Wnt proteins (2) and conveys them to the plasma membrane for secretion. Therefore, after secretion the lipid may be pivotal in limiting Wnt dispersion and its range of biological action, a precept to which we return below.

Once secreted, how Wnt signals are conveyed to their target cells remains cryptic. Some Wnt proteins may be incorporated into secretory vesicles (3), in which Wls continues to bind Wnt proteins (4) as a chaperone (Fig. 1B), perhaps availing the presentation of lipidated Wnt proteins to their cognate receptors, known as Frizzled receptors. Wnt signaling mediated by such vesicles would operate over a short distance, such as at the neuromuscular junction (4) and also in stem cell niches.

Although it is sometimes assumed that Wnt signals are long-range morphogens, there is little evidence that this is the prevailing mode of Wnt action. Wnt signaling occurs mostly between cells that are touching each other. Even in the

best studied example of long-range signaling by a Wnt—that is, by the Wnt ligand Wingless in *Drosophila*—recent evidence has made a case that the requirements for any function of Wingless can be largely afforded by a nondiffusible, membrane-tethered form of the protein (Fig. 1C) (5) and that Wingless does not act as a long-range morphogen in that context.

Once delivered to their target cells, Wnt ligands engage their cognate Frizzled receptors through their palmitate group, which extends into the lipid-binding cysteine-rich domain (CRD) of Frizzled receptors (6). Wnt ligands also bind the Lrp5/6 transmembrane co-receptor, inducing it to form a complex with Frizzled (Fig. 2A). This instills a conformational change in these receptors and enables phosphorylation by associated protein kinases. The phosphorylated cytoplasmic Lrp tail subsequently inhibits glycogen synthase kinase 3 (GSK3) (7) and also binds the Axin protein. In the absence of a Wnt signal, a destruction complex that includes Axin, anaphase-promoting complex (APC), and GSK3 phosphorylates β -catenin, continually targeting it for degradation by the proteasome. Inhibition of the destruction complex, a consequence of Wnt–Frizzled–Lrp interactions, leads β -catenin to accumulate in the nucleus (Fig. 2B). There, β -catenin governs transcriptional programs through association with Tcf/Lef transcription factors.

In some instances, Wnt signals are transduced independently of β -catenin—for example, during morphogenetic movements in vertebrate gastrulation (8). In this pathway, Frizzled and an intracellular transduction component (Dishevelled) are crucial, but not Lrp and β -catenin. This aspect of Wnt signaling is evolutionarily ancient and may be involved in regulating stem cell polarity and asymmetric division of stem cells within the confinement of the niche, as we discuss below.

Wnt signaling can be further augmented by secreted R-spondin proteins (9, 10). R-spondins, acting through Lgr family receptors (11–13), inhibit the transmembrane E3 ubiquitin ligases Rnf43/Znrf3 that ordinarily ubiquitinate and thus degrade Frizzled receptors (14, 15). By antagonizing Rnf43/Znrf3, R-spondins consequently stabilize surface Frizzled receptors and enhance Wnt signal strength (Fig. 2A) (14, 15).

The fundamental core of the Wnt pathway (Wnt, Frizzled, and downstream effectors) is evolutionarily ancient and is extant in the earliest multicellular animals including ctenophores, sponge, and placozoans (16, 17), in which it mediates basic axial patterning even in pre-bilateria (18, 19). In contrast, the R-spondin/Lgr axis is principally a vertebrate innovation (20). Was the R-spondin/Lgr pathway simply collateral to vertebrate speciation? Another possibility was that it was evolutionarily co-opted to amplify Wnt signaling and thus sustain some types of adult stem cell in long-lived vertebrate species (20).

Wnt-driven transcriptional programs

In the nucleus, β -catenin interacts with Tcf/Lef transcriptional cofactors to regulate the transcription of Wnt target genes (Fig. 2B). Rather than

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conforming to a universal program, the transcriptional agenda imposed by β -catenin varies between lineages. However, several generalities might exist. For instance, in Wnt-responsive stem cells it seems that β -catenin can directly induce telomerase expression (27), causally explaining the lengthy telomeres of Wnt-driven intestinal stem cells (22) and pluripotent cells (27) and shielding them from genomic catastrophe.

Although the phenotypic consequences of Wnt signaling diverge between distinct lineages, several genes appear to represent generic Wnt transcriptional targets. *Axin2* has emerged as one such Wnt target gene (23) that therefore serves as a reporter of ongoing Wnt signaling (24). As discussed below, *Axin2* (24) as well as a second gene, *Lgr5* (25), can identify Wnt-responding

lineages in diverse tissues. Genetically labeling *Lgr5*- or *Axin2*-expressing cells has revealed their participation in tissue renewal in multiple organs, compellingly nominating such cells as stem cells in specific tissues. We summarize these cell labeling experiments in Table 1 and discuss three examples in more detail.

Intestinal stem cells

The small intestinal epithelium is the fastest proliferating tissue of adult mammals, being largely made anew every 4 to 5 days (26). Villi protrude into the gut lumen and continually shed differentiated cells from their tips. These losses are replenished by stem cells located in proliferative intestinal crypts that surround the villus base (Fig. 3A). Wnt signals are pivotal

for the perennial renewal of the intestines, as shown by disruption of the pathway—which leads to the abrupt cessation of proliferation in the intestinal crypts, consequently leading to unabated loss of intestinal tissue and often morbidity (27–29). Reciprocally, the Wnt coagonist R-spondin potently stimulates intestinal proliferation in vivo (30).

The crypt bottom harbors slender, cycling “crypt base columnar” (CBC) cells (31), which were historically proposed to represent intestinal stem cells (32) (Fig. 3A). Exploiting the expression of Wnt target gene *Lgr5* in CBCs, genetic labeling of *Lgr5*⁺ crypt cells indeed demonstrated that these long-lived cells generate all differentiated intestinal cell types (25). Therefore, CBCs constitute multipotent intestinal stem cells (25) that require Wnt for proliferation (27, 33), perhaps explaining why Wnt is crucial for intestinal renewal.

Residing directly above the CBC stem cell zone at the “+4” position is a potentially distinct population of slowly cycling cells [variously described by molecular markers including *Bmi1* (34), *Hopx* (35), *Lrig1* (36, 37), and *Tert* (38, 39)] that also can generate all intestinal lineages (Fig. 3A).

Instead of constituting irrevocably separated lineages, it seems that *Lgr5*⁺ and +4 stem cells can interconvert. The highly proliferative *Lgr5*⁺ CBCs appear to be the “workhorse” of daily intestinal renewal (33). Yet, slowly cycling “reserve” +4 stem cells can be recalled to *Lgr5*⁺ status (40) and vice versa (35).

Adding further complexity, the two stem cell lineages may be partially overlapping. *Lgr5*⁺ cells can coexpress +4 markers (such as *Bmi1*) (41–43). Indeed, whereas the majority of *Lgr5*⁺ cells are proliferative stem cells, a subset of *Lgr5*⁺ cells are nondividing secretory precursors that coexpress +4 markers (43). These precursors, typically confined to secretory fates, can be promoted to multipotent stem cell status upon tissue damage to effect intestinal repair (43). This indicates that the developmental competence of precursors is not fixed but is rather labile, as we explore further below.

Interfollicular epidermis

The interfollicular epidermis (IFE) is constantly regenerated. Differentiated cells are shed from the surface and replaced by basal layer stem cells. Most basal layer cells transduce Wnt signals, as visualized by a Wnt transcriptional reporter and expression of Wnt target gene *Axin2* (44, 45). *Axin2*⁺ basal cells continuously produce keratinocytes for over 1 year in vivo and therefore qualify as IFE stem cells (Fig. 3B) (44, 45).

Certain evidence suggests that β -catenin is crucial for epidermal proliferation and maintenance of IFE stem cells, both in vivo (44–46) as well as in cell culture (47). However, extrapolating a role for Wnt as an IFE self-renewal signal based on these data has been complicated by the fact that β -catenin operates dually in cell adhesion (48) as well as Wnt/ β -catenin signaling. Implying a role for Wnt signaling specifically, simultaneous loss of *Tcf3* and *Tcf4* compromises

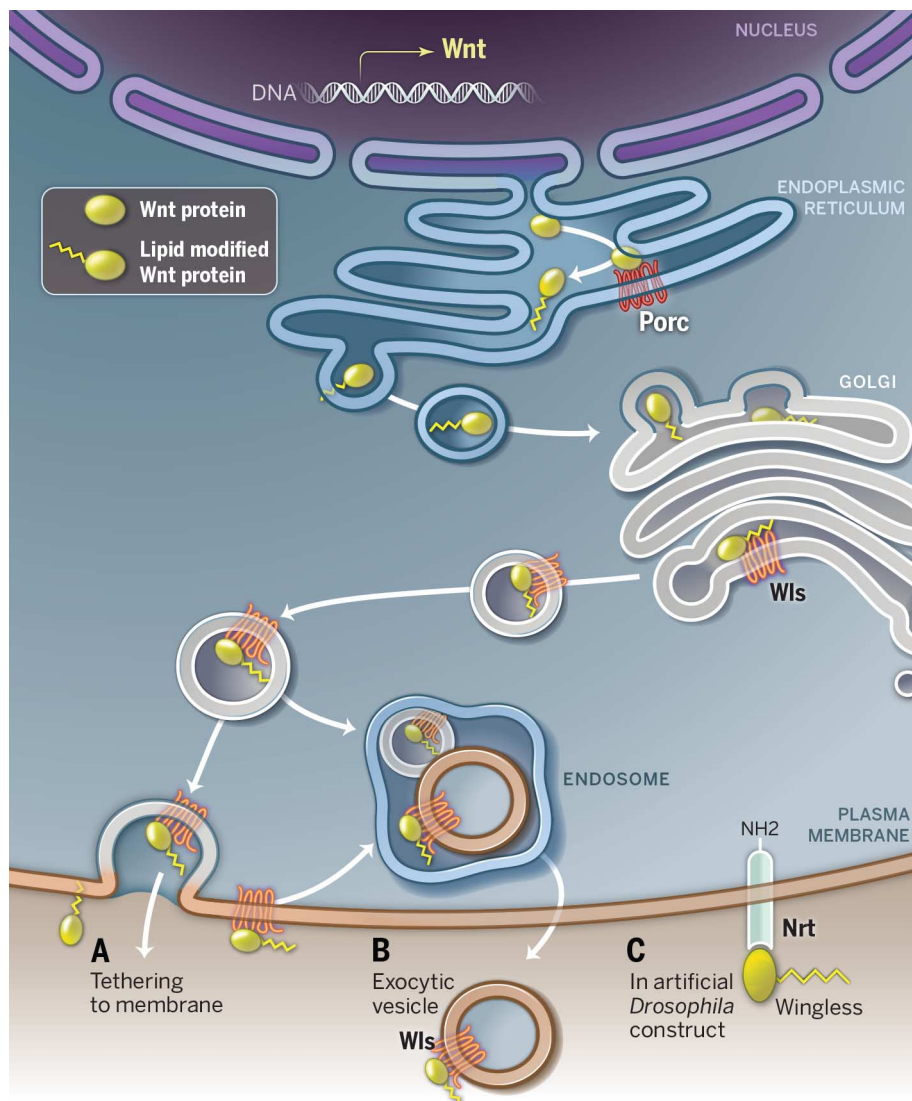


Fig. 1. Model of Wnt secretion, modification, and short-range signaling activity. Wnt proteins are lipid-modified by the Porcupine enzyme in the endoplasmic reticulum. Subsequently, lipid-modified Wnts are bound by the carrier protein Wls and might be expelled in (B) secretory vesicles furnishing membrane-bound Wnt ligands or (A) might be directly presented as cell surface-bound Wnt ligands. (C) In *Drosophila*, a constitutively membrane-tethered Neurotactin (Nrt)–Wingless fusion protein is able to execute all Wingless functions, implying that Wnts need not be released from the membrane in order to signal.

long-term IFE maintenance (49). Taken in collective, these findings suggest that IFE basal stem cell proliferation is controlled by Wnt signaling. Furthermore, basal cells produce their own Wnt ligands (44), implying autocrine (rather than niche-dependent paracrine) regulation (Fig. 3B). This concept portends a type of “developmental self-organization,” considered further below.

Mammary gland

The mammary gland constitutes another venue of tissue renewal because it undergoes cycles of dynamic growth during puberty, pregnancy, and lactation. After lactation, the alveoli in the gland regress by involution and cell death, and the tissue returns to a pre-pregnancy-like state. How are these cycles of regrowth continually sustained?

Initial transplantation (50) and subsequent lineage-tracing experiments have established that stem cells exist in the adult mammary epithelium that and they appear to be driven by Wnt signaling (51) because they are designated by *Lgr5* (52–55) and *Axin2* (24) in vivo and can be expanded in vitro upon Wnt exposure (56). *Axin2*⁺ cells self-renew and continuously fuel cellular production during multiple cycles of pregnancy, lactation, and involution (24), indicating that these cells (or a subset of them) are authentic stem cells.

Stochastic fate or invariant lineage?

The classical view of homeostatic stem cell self-renewal is exemplified by that of the hematopoietic stem cell, which is believed to divide rarely and invariably in an asymmetric fashion to generate one new stem cell and one differentiated daughter. However, neither the intestinal crypt nor the IFE abide by this rule of predetermined lineage choice. Each crypt contains a fixed number of stem cells, determined by the size of the niche. Each of these stem cells divides every day to generate two new “potential” stem cells. Chance decides which of these will stay within the niche at the crypt bottom and which are pushed out of the niche (57, 58). This process is termed “neutral competition” and ensures that (i) the number of available stem cells is constant and (ii) that damaged or lost stem cells are immediately replaced by healthy neighbors (59). Also in the skin, the Wnt-responding IFE stem cells appear to divide stochastically to generate proliferating and differentiating daughter cells with equal probability (44, 60). Thus, whether any given stem cell daughter will continue self-renewing is left to a throw of the dice—not destiny.

Plasticity within the stem cell hierarchy

In models of the hematopoietic hierarchy (61), all arrows “point away” from the stem cell, implying that once cells give up their stem cell identity, there is no way back. Intestinal cells do not abide by this rule. Although Dll1⁺ secretory progenitors are typically short-lived precursors that are confined to secretory fates (Fig. 3A), if crypt stem cells are depleted, Dll1⁺ secretory progenitors can regain Lgr5⁺ stem cell status in vivo

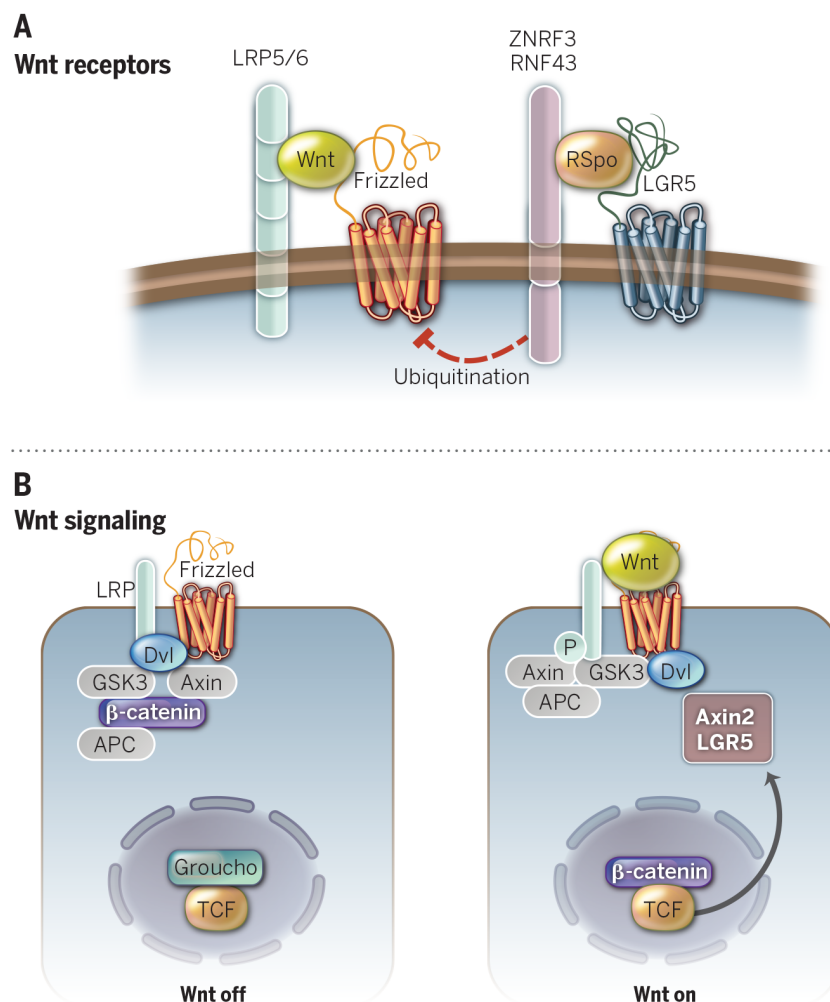


Fig. 2. Wnt signaling mechanisms. (A) Wnt reception on the cell surface. Wnt ligands bind to the Frizzled and Lrp5/6 receptors, activating downstream signaling. The membrane proteins Znr3 and Rnf43 are ubiquitin ligases that continually down-regulate Frizzleds through ubiquitination. Binding of R-spondins to Znr3 and Rnf43 and the Lgr4/5/6 receptor relieves Znr3 and Rnf43 activity, thus stabilizing Frizzleds. (B) Wnt signaling in target cells. (Left) In the absence of Wnt, a destruction complex consisting of Axin, APC, and GSK3 resides in the cytoplasm, where it binds to and phosphorylates β -catenin, which is then degraded. Dvl (Disheveled) is required for activating the pathway as well. In the nucleus, T cell factor (TCF) is in an inactive state as the consequence of binding to the repressor Groucho. (Right) Binding of Wnt to its receptors induces the association of Axin with phosphorylated lipoprotein receptor-related protein (LRP). The destruction complex falls apart, and β -catenin is stabilized, subsequently binding TCF in the nucleus to up-regulate target genes, including *Axin2* and *Lgr5*.

Table 1. Wnt-responsive tissue stem cells identified by means of lineage tracing.

Tissue	Stem cell	Marked by	Reference
Intestine	Crypt base columnar cell	Lgr5	(25)
Mammary gland	Basal cell	Axin2, Lgr5	(24, 50–53)
Stomach	Basal pyloric cell	Lgr5	(85)
Interfollicular epidermis	Basal cell	Axin2	(44, 45)
Central nervous system	Radial glial cell	Axin2	(98)
Hair follicle	Outer bulge cell	Lgr5	(99)
Kidney	Nephron segment-specific stem cell	Lgr5, Axin2	(100, 101)
Cochlea	Tympanic border	Axin2	(102)
Ovary	Hilum ovarian surface epithelial cell	Lgr5	(103)
Taste bud	Circumvallate papilla stem cell in posterior tongue	Lgr5	(104, 105)

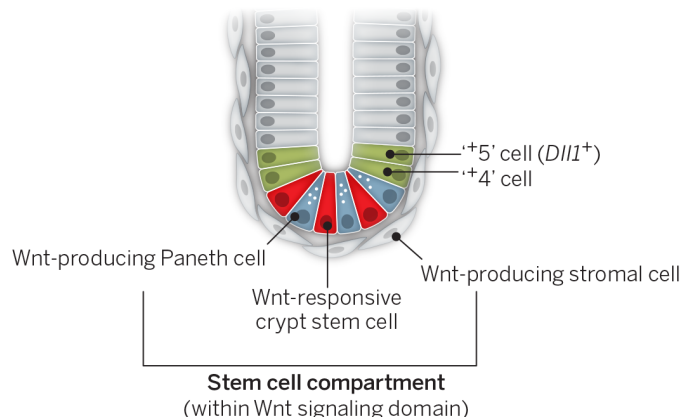
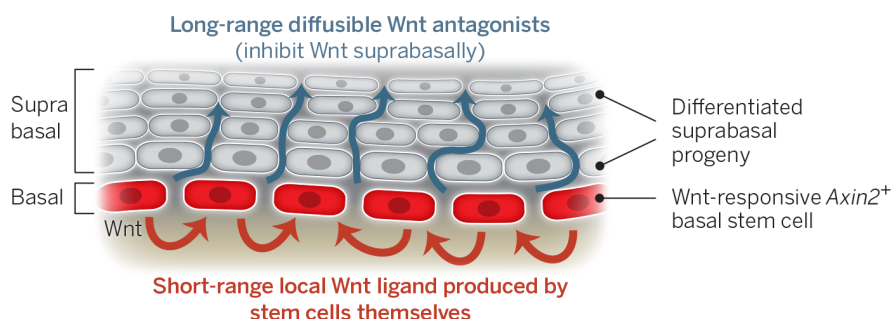
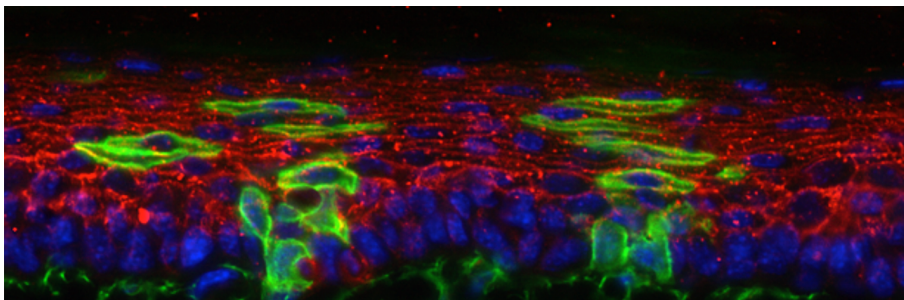
A**External niche: intestinal stem cells within the crypt****B****A niche within: epidermal stem cells produce their own Wnt****C****Epidermal stem cells produce Wnts and Wnt inhibitors**

Fig. 3. The provenance of Wnt ligands in the stem cell niche. (A) At the intestinal crypt bottom, Paneth cells and stromal cells supply Wnt ligands to sustain the self-renewal of $Lgr5^+$ crypt stem cells, with which they are intercalated. The local Wnt signaling domain spatially delimits stem cell activity to the crypt bottom. Cells moving upward begin to differentiate, although they may be restored to stem cell status upon returning to the crypt bottom. (B) Within the interfollicular epidermis, basal-layer stem cells express Wnt ligands and thus continuously induce their own self-renewal and act as their own niche. Basal stem cells also express long-range Wnt antagonists that diffuse to suprabasal layers, basally limiting the Wnt signaling field and “self-organizing” the stratified epidermal architecture. (C) Image of Dkk3 immunostaining (red) in epidermis of $Axin2$ -CreERT2/ $Rosa26$ -mTmG mice exposed to Tamoxifen at P21 to induce labeled clones (green) and chased for 2 months (P77). (C) is courtesy of X. Lim (44).

(62). In vitro, this process can be mimicked by a pulse of high-dose Wnt3a (62). Similar observations were reported for a noncycling secretory precursor (43). Therefore, lineage-restricted progenitors may gain an expansion of responsibility upon injury, reacquiring multipotency and long-term self-renewal to perpetuate tissue repair. The stem cell phenotype is not indelibly imprinted but may be ordained unto other cell types during the regenerative response.

Wnt and tissue regeneration in the earliest animals

Even in the earliest animals, it seems that Wnt coordinates repair after injury in certain tissues and imparts positional information crucial for shaping proper regeneration. Upon resection of their tail, planarian flatworms regenerate their tail anew. Nonetheless, upon depletion of β -catenin, a head is inappropriately regenerated in lieu of the tail, leading to the generation of multiple heads (63, 64). Therefore, Wnt ensures that the original anatomic plan is faithfully restored after injury. Analogously, *Wnt10a* is up-regulated upon zebrafish tail resection and is necessary for robust tail regeneration (65). Likewise, *Wnt3* is crucial for apical regeneration of amputated hydra (66). Compellingly, in hydra the Wnt source is apoptotic cells at the site of the wound, which provide Wnt3 to drive proliferation of underlying cells and thus regeneration (67). Therefore, Wnt elegantly links tissue loss with how such tissue might be restored.

The sources of Wnt ligands: Redefinition of the stem cell niche

Wnt signals, by virtue of their short-range nature, constitute ideal “niche factors,” controlling immediately adjacent stem cells and thus permitting parsimonious command of cell fate.

For instance, $Lgr5^+$ CBCs in the crypt bottom are evenly interspersed with Paneth cells (68) that, together with nonepithelial lineages including mesenchymal cells (69–71), supply Wnt proteins to maintain adjacent $Lgr5^+$ CBCs (Fig. 3A). The localized spatial reach of Wnt dictates that only cells near the crypt bottom remain stem cells. Cells migrating upward out of the reach of Wnt signaling differentiate.

This “Wnt-adjacency” model can also hold true in regeneration. Upon bladder injury, stromal cells directly underlying the bladder basal epithelium up-regulate Wnt ligands, signaling to adjacent basal stem cells to initiate bladder epithelium regeneration (72). Therefore, stem and niche cells are paired in both spatial location and function.

Nevertheless, the past few years have seen a revision to the monolithic notion that stem cells need always be controlled by an extrinsic niche. $Axin2^+$ IFE stem cells express their own Wnt ligands, which they require for self-renewal (44). Therefore, they may continuously drive their own self-renewal in an autocrine fashion (Fig. 3B), akin to how Wnt3a-expressing axial stem cells in the early vertebrate embryo in essence act as their own niche (73) to sustain their own self-renewal during axis elongation and upon

serial transplantation (74, 75). In the case of the intestinal crypt, *Lgr5*⁺ CBCs generate Wnt-producing Paneth cells (25). This underpins why single *Lgr5*⁺ CBCs can form intestinal organoids in vitro in the absence of niche cells (76)—because stem cells can elaborate their own niche.

Developmental self-organization

These observations imply that in some contexts, stem cells can self-organize their own niche and autonomously perpetuate their activity. In this capacity, stem cells qualify as fundamental “units of development” (61) because they can incipiently seed developing tissues anew. In the developing *Drosophila* intestine, the first cell division undertaken by the earliest intestinal stem cells is to asymmetrically generate a niche cell as well as another stem cell (77). “Auto-niche generation” enables single stem cells to take root in the nascent tissue, expand to form islands of undifferentiated stem cells, and subsequently fuel intestinal development (77).

If stem cells can self-organize their own niche and continue ever-expanding in vivo, this could be easily subverted to lead to tumorigenesis. Contrary to this notion of unchecked stem cell expansion, in each intestinal crypt there exists approximately 14 *Lgr5*⁺ CBCs and 10 Paneth cells per crypt bottom (57) and eight *Lgr5*⁺ stem cells per stomach pylorus pit (78). How is stem cell expansion so precisely constrained in the steady state? By way of example, in the skin, IFE basal stem cells produce not only their own Wnt ligands but also diffusible Wnt antagonists, including Dkk molecules (Fig. 3C) (44). Therefore, adjacent basal stem cells signal via Wnt to sustain one another in the basal compartment, yet Dkk diffuses to the suprabasal layer to limit the Wnt signaling field and likely to induce differentiation in that domain (44). Consequently, stem cell activity is spatially confined to the basal layer, and Dkk might prevent expansion of the stem cell territory beyond that layer (Fig. 3B). In so doing, IFE stem cells might self-organize the stratified architecture of the epidermis.

Orienting asymmetric stem cell divisions by Wnt signaling within the niche

Stem cell numbers also may be numerically limited within the niche by Wnt-imposed asymmetric

stem cell divisions. *Drosophila* germline stem cells divide next to neighboring hub cells. The daughter cell closest to the hub cell remains a stem cell, whereas the distal cell invariably differentiates; this asymmetric division is oriented by the Wnt signaling component APC (79). Experiments using a local source of Wnt in cell culture imply a conserved mechanism extending to mammals. A localized Wnt signal can orient a mouse embryonic stem cell (ESC) to divide asymmetrically by placing the centrosomes at opposite ends of the cell, thus orienting the mitotic spindle of the dividing cell (Fig. 4) (80). This generates a Wnt-proximal and Wnt-distal daughter cell, the latter out of contact with the signal. In the Wnt-proximal cell, Wnt signaling maintains the stem cell fate, whereas the distal daughter differentiates (80). The orientation of stem cell division is therefore coupled with the position and fate of the dividing cell through the same signal. Therefore, in some tissues Wnt signals may orient stem cell divisions within the niche in an asymmetric fashion, delimiting stem cell number and ensuring a proper ratio of stem cells to their committed progeny.

Growing Wnt-dependent stem cells

The roles of Wnt in stem cell self-renewal or lineage-specific differentiation in diverse tissues in vivo are manifold; therefore, Wnt signals have found practical use in manipulating stem cell developmental programs in vitro. From a pragmatic perspective, because Wnt induces stem cell self-renewal in certain organs, it enables the in vitro propagation of such cells. For example, mammary gland stem cells can be expanded in vitro in the presence of Wnt protein and retain their ability to reconstitute the entire mammary organ after transplantation (56).

Similarly, pluripotent naïve ESCs from the rodent blastocyst may be cultivated in vitro in defined conditions by combining Wnt agonists [either Wnt protein (81) or GSK3 inhibitors (82)] with either leukemia inhibitory factor (LIF) signals or mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) inhibitors (83), as exemplified by the “2i” culture regime for serum-free ESC culture (82).

Because of the primacy of Wnt in instructing the intestinal stem cell fate, *Lgr5*⁺ CBC stem

cells can be expanded in an R-spondin1-based three-dimensional culture system in ever-growing organoids, or “mini-guts” (76), in which crypt and villus domains are established containing normal ratios of the appropriate cell types, whereas self-renewal kinetics closely resemble the in vivo situation (84). Comparable protocols have been established for *Lgr5*⁺ cells derived from the stomach (85), liver (86), and pancreas (87). When cells within organoids produce Wnt (for example, Paneth cells that secrete Wnt3 in small intestinal organoids), the addition of R-spondin suffices. When organoids harbor no endogenous source of Wnt (for example, colon organoids), exogenous Wnt3a is added in addition to R-spondin (88). Transplantation of clonal (single *Lgr5*⁺ stem cell-derived) organoids derived from colon and liver has confirmed that the cultured organoids retain their physiological functions (86, 89). This again provides evidence for substantial developmental self-organization—namely, that single *Lgr5*⁺ intestinal stem cells carry the morphogenetic information to create a structured tissue of complex architecture and diverse lineages.

Proper lineage differentiation and crypt-villus organization within small intestinal organoids relies on an interesting property of R-spondin1. Namely, it augments preexisting domains of Wnt signaling in the crypt bottom (68) rather than inducing Wnt signaling de novo. Thus, when cells exit the crypt bottom-like structures of mini-guts and the spatial reach of Wnt, intestinal differentiation occurs normally (76), accounting for proper organoid architecture. In contrast, spatially uniform Wnt activation by GSK3 inhibition captures a rather homogeneous population of *Lgr5*⁺ stem cells in vitro in the absence of differentiated lineages (90).

That being said, Wnt does not ubiquitously instruct stem cell self-renewal and, in multiple cases, instead drives differentiation—for instance, Wnt instead stimulates primed pluripotent stem cells (including human ESCs) to differentiate into primitive streak (91, 92).

Concluding remarks

The emergent view is that lipid-modified Wnt signals predominantly act over short ranges to locally control cell behavior, economically controlling stem cells within the spatial confines of

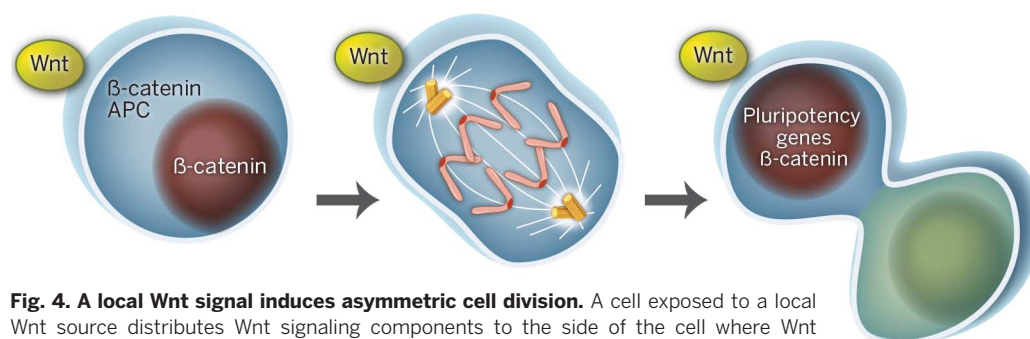


Fig. 4. A local Wnt signal induces asymmetric cell division. A cell exposed to a local Wnt source distributes Wnt signaling components to the side of the cell where Wnt touches. This orients the mitotic spindle and centrosomes during division. The daughter cell close to the Wnt source maintains nuclear β -catenin and stem cell gene expression, whereas the distal cell away from Wnt loses expression of such genes.

the niche. The short range of Wnt action implies a parsimonious model of niche organization and tissue physiology. Namely, in particular tissues it seems that Wnt-dependent stem cells are spatially restricted to the vicinity of the Wnt-producing niche, physically delimiting the stem cell compartment and preventing unauthorized stem cell expansion. When a stem cell divides, chance may dictate which (if any) of its successors are ousted from its niche, as in the intestines (57), stomach (78), and skin (44). In other lineages, Wnt itself may orient stem cells to divide asymmetrically (80), conveniently

anchoring Wnt-proximal stem cells to the niche and ensuring proper spatial allocation of stem cells and differentiated progeny.

In certain organs, stem cells exiting the niche become deprived of Wnt and therefore differentiate. Nonetheless, developmental plasticity may yet remain because early committed precursors can flexibly regain stem cell status upon tissue damage in vivo (43, 62, 93, 94) or Wnt3a treatment ex vivo, in some instances (62). This is profound because it indicates that lineage potential is an amorphous property in vivo; lineage-restricted precursors can gain an expansion of responsibility upon injury and become fully fledged multipotent stem cells once more. Intravital microscopy has documented that upon intestinal or hair follicle damage, precursors are spatially recalled to the stem cell niche (95, 96), upon which they reenter the niche signaling domain and presumably become promoted to stem cell status as a consequence, although the responsible signals remain largely elusive. Therefore, lineage barriers between stem cell and progenitor states are not always stringent in vivo and can be traversed during times of tissue damage and repair (43, 62, 93, 94). If stem cell and progenitor fates are interconvertible upon niche contact (97), then stem cell status might not be an intrinsic entitlement but rather a positional privilege—reflecting whether a cell is currently in the embrace of the niche.

Nonetheless the notion of a “niche” must be refined because some stem cells may act as or establish their own niche ab initio, portending unexpected developmental self-organization. Such intrinsically programmed stem cell behavior could underpin emergence of complex patterned tissues during development and/or regeneration, as in the *Drosophila* (77) and mouse (76) intestines.

The above findings identify an integral program for tissue generation, regeneration, and renewal. In evolutionary antiquity, the core of the Wnt pathway emerged in the simplest multicellular organisms (16, 17). Accruing evidence suggests that in the earliest metazoa, Wnt was an ancestral “symmetry-breaking” signal that separated otherwise-symmetric embryos into two halves (the anterior versus the posterior domain) and in so doing enabled the evolutionary emergence of axially patterned animals (18, 19). Simply put, the primordial role of Wnt signaling in the earliest animals was pattern formation (during tissue generation) and pattern maintenance (during tissue regeneration), as evinced by how Wnt establishes a bodily pattern in hydra and planaria and enables the reconstitution of such pattern upon tissue regeneration (63, 64, 66). In long-lived vertebrates, this ancestral pattern maintenance program has since been extended to tissue renewal, in which Wnt permits several tissues, including the skin and intestines, to be continuously replenished and thus maintained over a lifetime.

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RESEARCH ARTICLE

PROTEOMICS

Tracking cancer drugs in living cells by thermal profiling of the proteome

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The thermal stability of proteins can be used to assess ligand binding in living cells. We have generalized this concept by determining the thermal profiles of more than 7000 proteins in human cells by means of mass spectrometry. Monitoring the effects of small-molecule ligands on the profiles delineated more than 50 targets for the kinase inhibitor staurosporine. We identified the heme biosynthesis enzyme ferrochelatase as a target of kinase inhibitors and suggest that its inhibition causes the phototoxicity observed with vemurafenib and alectinib. Thermal shifts were also observed for downstream effectors of drug treatment. In live cells, dasatinib induced shifts in BCR-ABL pathway proteins, including CRK/CRKL. Thermal proteome profiling provides an unbiased measure of drug-target engagement and facilitates identification of markers for drug efficacy and toxicity.

The complete determination of the proteomic state of a cell, referred to as the proteotype, links genotype and the cellular phenotype. Thus, the proteotype of the target cell or tissue represents an important context for the study of drug action. However, the task of accurately describing a proteotype remains daunting because of the inherent complexity of the proteome, comprising in excess of 10,000 gene products expressed at levels differing over six or more orders of magnitude, many of which occur in different splice isoforms and with different posttranslational modifications at different subcellular localizations (1). Current expression proteomics methods describe proteotypes as lists of proteins and semiquantitative expression levels (2). In addition, there are methods for the cell-wide assessment of some but not all posttranslational modifications (3, 4). Studies focused on the mechanism of bioactive compounds, such as small-molecule drugs or peptides, frequently rely on affinity-based enrichment strategies to identify prospective binding partners from a cell extract (5–7). These approaches can be combined for the differential study of cellular phenotypes—for instance, the status of signaling pathways or for biomarker discovery (8, 9). However, unbiased

approaches for the cell-wide assessment of protein state and protein function are not available.

Changes in the thermal stability of proteins are frequently used to study ligand binding (10, 11) and, with the recent development of the cellular thermal shift assay (CETSA), can now be observed in living cells (12), enabling the monitoring of target engagement, which is a key parameter in drug discovery (13). We extended this approach to address two challenges critical in drug discovery: target and off-target identification and discovery of molecular biomarkers for drug efficacy. By combining the CETSA method with multiplexed quantitative mass spectrometry (MS), we established the proteome-wide determination of protein thermal stability in intact cells as an independent and complementary strategy for the characterization of cellular proteotypes. Our approach, termed “thermal proteome profiling,” includes but is not limited to the monitoring of protein-ligand interactions directly in cells or tissues because in theory, any modification of a protein can affect its thermal stability (14). Here, we demonstrate that monitoring thermal stability across cellular proteomes in different states, such as under drug treatment, enables the identification of direct physical interaction partners and downstream effectors as markers of target engagement and drug efficacy.

Proteome-wide profiling of protein thermal stability

To monitor the thermal stability of proteins across 10 different temperatures, we used the recently developed neutron-encoded isobaric mass tagging reagents (TMT10) (15) in conjunction with high-resolution MS. This allowed acquisition of full melting curves for a large proportion of expressed soluble proteins in a single liquid chromatography–

MS (LC-MS)/MS experiment. In a typical experiment, cells were cultured under differential conditions, such as drug treatment (Fig. 1). For each condition, the cells were divided into 10 aliquots, each of which was briefly heated to a different temperature followed by extraction with phosphate-buffered saline (PBS). Around their intrinsic melting temperature, proteins in the cell denature and subsequently aggregate (16), resulting in their gradual disappearance from the PBS-extracted samples with increasing temperatures (12, 17). This protocol is only suitable for the soluble fraction of the proteome because membrane proteins are not solubilized under these conditions. After extraction, each sample was trypsinated and labeled with a different isotope-coded isobaric mass tag (15), and the 10 samples from each condition were mixed and analyzed by means of LC-MS/MS. The reporter ion intensities acquired in the MS/MS fragment spectra were used to fit a curve and calculate a specific melting temperature (T_m) for each protein, which was then compared between the vehicle-treated and drug-treated samples. The resulting curves that display the increase in protein aggregation with temperature in many cases directly reflect the underlying unfolding or “melting” event. For many proteins, a good correlation between the thermofluor unfolding assay and heat-induced precipitation in solution, or in cells by CETSA, has been demonstrated (11, 17), suggesting that properly shaped curves represent real unfolding events. Hence, ligand-induced curve shifts indicate a change in the thermal stability of the protein rather than a change in the aggregation properties. In a variation of the cell-based protocol, thermal stability can also be assessed in a cell extract. This alternative strategy avoids the separate extraction of each cell sample, potentially allows more controlled conditions (12), and in conjunction with cell treatment experiments, enables distinguishing T_m shifts induced by ligand binding from those induced by downstream modifications.

The thermal profile of a cellular proteome

We acquired quantitative thermal stability data for 5299 proteins across 10 different temperatures from the human K562 chronic myeloid leukemia cell line. This data set could be regarded as the first description of the melting proteome (“meltome”) of a human cell. Thermal profiles were acquired in two experimental settings in which either intact cells or cell extracts were heated. In both settings, we noted a weak but significant anticorrelation of the thermal stability with molecular weight because smaller proteins tend to be more stable (fig. S1). A comparison of the thermal stability across a set of 3204 proteins robustly quantified in both intact cells and cell extract revealed marked differences in melting properties between cells and extract (Fig. 2 and table S1). Hierarchical cluster analysis of the temperature-dependent relative protein concentrations in the heated cell samples revealed a group of proteins that exhibited an increase in concentration at ~50°C, followed by a pronounced decrease at ~56°C, and another group of proteins with a concentration increase at ~63°C.

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The concentration increases observed at these temperatures indicate increased solubilization at the initial temperatures followed by aggregation at higher temperatures. Gene ontology analysis indicated that the proteins in these clusters are released from disintegrating organelles (such as mitochondria) or large protein assemblies (such as ribosomes) (Fig. 2A and fig. S2). When cell extract rather than intact cells was heated, we did not observe any substantial increases in protein concentration by heating, likely because of the disruption of cellular structures during cell lysis. Melting points were determined for proteins passing curve-fitting quality criteria as described in the supplementary materials, materials and methods (Fig. 2B). On average, proteins showed 2.7°C higher T_m values in cell extract as compared with intact cells. Although this difference is significant (SEM = 0.06°C, $P < 0.01$, t test), the correlation of melting points acquired in cell extracts and intact cell experiments ($R^2 = 0.3$) is considerably lower than the correlation of the respective replicate experiments (intact cell, $R^2 = 0.93$; cell extract, $R^2 = 0.89$), suggesting that the disruption of the cellular context heterogeneously affects protein stability (Fig. 2B). The global trend of increased protein stability in cell extract suggests an effect on protein precipitation due to lower protein concentrations in cell extract as compared with intact cells. This contrasts with the hypothesis that molecular crowd-

ing in the cytosol should exert a stabilizing effect, which was based on the observation that phosphoglycerate kinase exhibits increased stability inside the cell (18). This is in line with our data for this protein but could also be explained by a stabilizing effect of the endogenous cosubstrate adenosine 5'-triphosphate (ATP) (fig. S3). We speculated that proteins that are bound to a ligand inside the cell might show an increase in thermal stability contrary to the general trend because the dissociation of protein-ligand complexes during extraction would result in their destabilization. This hypothesis was substantiated by an analysis of the T_m values of a set of 440 proteins annotated as ATP binders, which showed a significant trend ($P < 0.01$, t test) toward increased stability in intact cells when compared with all other proteins (table S2). This was experimentally confirmed with the determination of thermal shifts induced by the addition of MgATP to a K562 cell extract at approximately physiological concentrations (2 mM) (19). In these thermal profiles, we detected 213 proteins annotated as ATP binders that in two independent replicates showed the predicted trend toward increased stability upon addition of ATP, whereas all other proteins detected in these experiments did not show this trend (fig. S4 and table S3). Further analysis of the thermal proteome profiles revealed that proteins categorized as DNA binders were enriched in the set with the lowest

melting points, suggesting that these proteins were extracted without their DNA ligand. Indeed, the DNA-binding properties of proteins can be assessed by adding DNA fragments to the cell extract. This is exemplified by p53, the global transcription regulator for stress. Wild-type p53 was stabilized upon addition of its cognate effector DNA, whereas the p53 R273H mutant that does not bind these effector sequences (20) was not stabilized (fig. S5). These results demonstrate the potential of thermal proteome profiling for the large-scale analysis of proteome-ligand interactions, including endogenous ligands such as cofactors or metabolites.

Monitoring of drug effects on thermal proteome profiles

The analysis of drug mechanism of action by profiling both targeted and off-target protein binding will likely represent a major application of thermal proteome profiling. As a proof of principle, we studied kinase inhibitors because kinases are an important target class, with key regulatory functions in cellular pathways. An important question in drug target identification is the prevalence of false-positive and false-negative identifications. To investigate the reliability of thermal proteome profiling, we selected two structurally divergent promiscuous kinase inhibitors with a known spectrum of targets, staurosporine and GSK318257L, which is a close analog of CTx-0294885 (21). Profiles

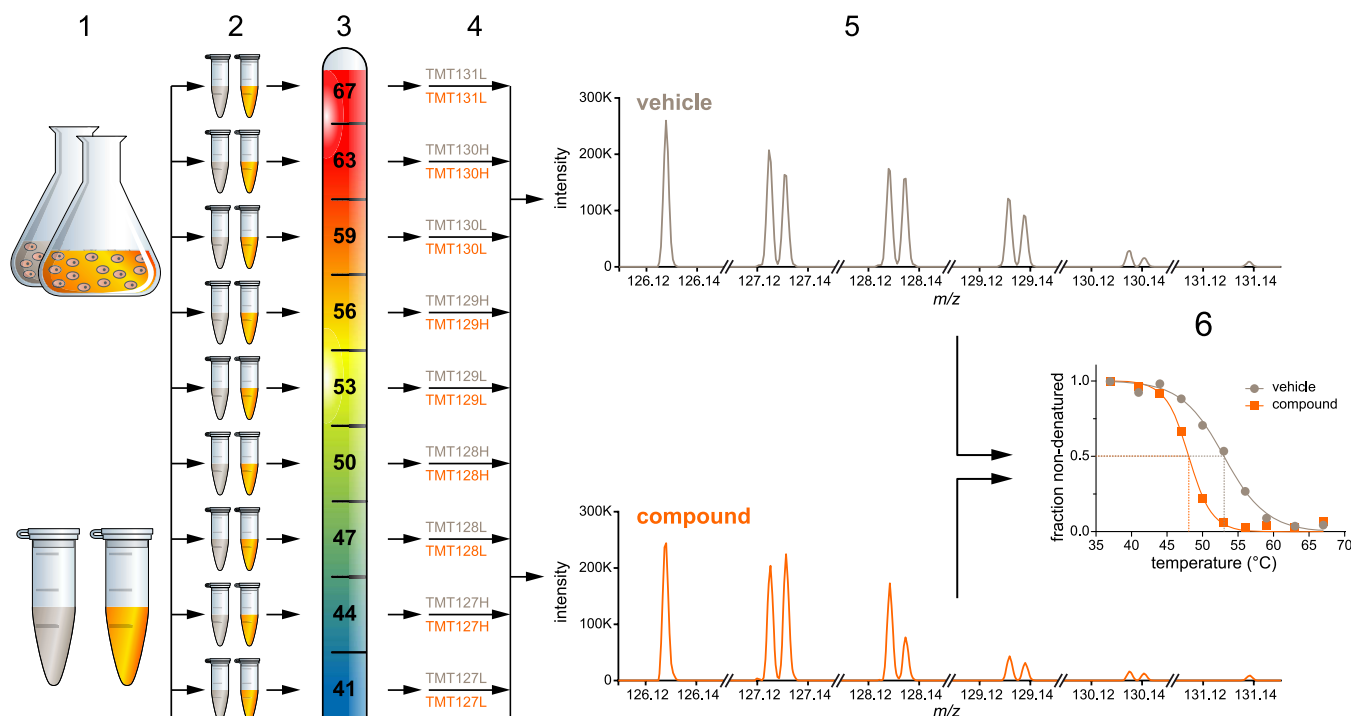


Fig. 1. Quantitative proteome-wide profiling of protein thermal stability under differential conditions. Cells were cultured under differential conditions, such as drug treatment. In an alternative method, the cells were extracted first, and the extracts were treated with drug ("1").

For each condition, the cell or cell-extract sample was divided into 10 aliquots ("2"). Aliquots were subjected to heating at the indicated temperatures. Samples of intact cells were subsequently subjected to extraction with PBS ("3"). After digestion with trypsin, each sample was labeled with a different TMT10 isotope tag ("4"). Subsequently, all samples from each condition were mixed ("4") and analyzed by means of LC-MS/MS ("5"). The obtained reporter ion intensities were used to fit a melting curve and calculate the melting temperature T_m of each protein separately for the two conditions ("6").

of K562 cell extract treated with staurosporine or vehicle were generated as two independent replicates (Fig. 3). Of all proteins, 92% detected yielded curves with sufficiently steep slopes to allow the robust identification of ligand-induced T_m shifts. However, a shallow slope of the melting curve indicated lower melting point reproducibility (Fig. 3A). Whereas most affected proteins showed positive shifts consistent with ligand-

induced stabilization, a few proteins, including members of the protein kinase C family, exhibited negative shifts, which may result from destabilization. In total, the thermal profiles comprised 175 protein kinases, of which 51 displayed T_m shifts that passed our significance criteria (Fig. 3B, fig. S6, and table S4). A recent comprehensive analysis of staurosporine targets identified by means of chemoproteomics “kinobeads” profil-

ing (22) detected 229 kinases, of which 92 were also present in both biological replicates of the thermal profiles experiments. The limited degree of overlap may be due to the fact that kinobeads will fail to identify kinases that do not bind to the immobilized ligands, whereas thermal profiling will miss proteins owing to insufficient abundance and/or solubility or the absence of a significant ligand effect. Analysis of the 92 kinases common to both data sets revealed that kinobeads identified 66 kinases with a staurosporine median inhibitory concentration (IC_{50}) below $10\ \mu\text{M}$, of which 34 showed a significant T_m shift, and an additional 15 showed a reproducible shift of greater than 1°C , which could become significant with additional replicates. For some proteins, we observed shallow or irregular curves (for example, BMP2K, AAK1 in fig. S6, G and J, respectively) that may reflect multiple transitions possibly because of independent folding domains and are difficult to fit with our current methods. For the remainder of 17 kinases identified as targets by kinobeads, we observed very small ($<1^\circ\text{C}$) or no T_m shifts (Fig. 3C). These targets may represent false negatives. We also observed thermal shifts for proteins other than kinases. Staurosporine induced apparent stabilization of coproporphyrinogen-III oxidase and ferrochelatase (FECH), two out of the eight enzymes in the heme biosynthesis pathway, for all of which melting curves were obtained (Fig. 3D and fig. S7). Ligand-binding also affected the thermal stability of the regulatory components of some protein complexes—for instance, kinase complexes containing cyclins (fig. S6, C and J). We further explored this in the context of the protein kinase A (PKA) complex (23). Inhibition by staurosporine appeared to stabilize the catalytic subunit but destabilize the regulatory subunit. In contrast, the addition of adenosine 3',5'-monophosphate (cAMP), which causes dissociation of the regulatory subunit from the catalytic subunit, appeared to stabilize the regulatory subunit but destabilize the catalytic subunit, presumably because of the release of the regulatory subunit (Fig. 3E).

Comparison of staurosporine T_m shift data with kinobeads-derived potencies for multiple targets (Fig. 3C) showed no significant correlation between the affinity for staurosporine and the magnitude of the T_m shift. This is not surprising because the extent of thermal stabilization by ligands with a similar binding mode likely depends on the stability of the unliganded protein. To investigate the correlation between ligand affinity and apparent thermal stabilization, we considered a structurally divergent promiscuous kinase inhibitor, GSK3182571 (Fig. 4 and table S5). We identified 13 targets common to both inhibitors with an affinity below $1\ \mu\text{M}$ and a less than 10-fold difference in affinity. For these targets, both compounds showed similar T_m shifts ($R^2 = 0.9$) (Fig. 4A), suggesting that for a given protein, the T_m shift at saturating ligand concentrations is dependent on the intrinsic affinity of the ligand.

Depending on the number of replicate experiments, ligand-induced T_m shifts as small as 1°C can be monitored. However, to resolve small

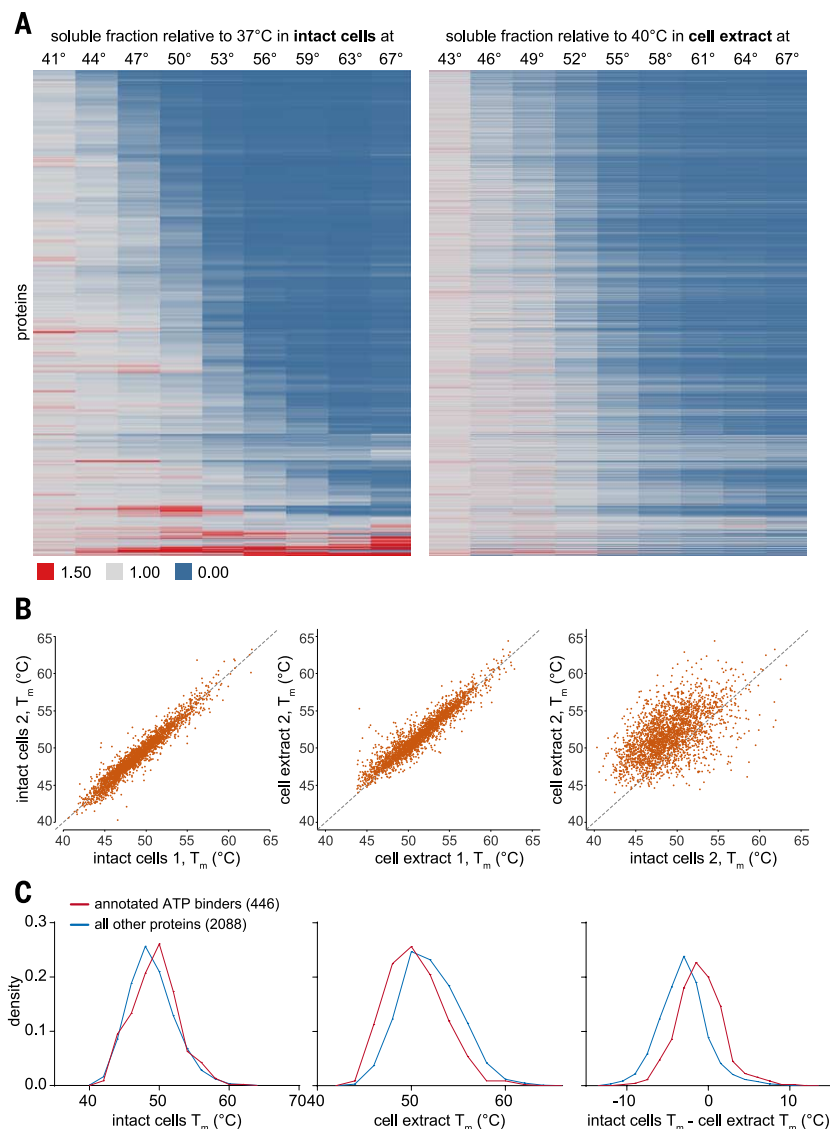


Fig. 2. The thermal proteome profile of a human K562 cell. (A) Heat map representation of the thermal stability of 3204 soluble proteins in intact cells (left) and cell extract (right). For each protein, its relative concentration (fold-change) at the indicated temperature compared with the lowest temperature (37°C for intact cells and 40°C for the cell extract) is shown. Protein fold-changes from the experiment on intact cells were clustered. To allow better comparison, proteins heated in the cell extract were plotted in the same order as the proteins heated in intact cells. (B) Reproducibility of thermal proteome profiles assessed with replicate experiments on intact cells, $R^2 = 0.93$ (left), cell extract, $R^2 = 0.89$ (middle), and direct comparison of proteomes from intact cells and cell-extract experiments, $R^2 = 0.30$ (right). Most proteins showed greater thermal stability (higher T_m values) in cell extract as compared with intact cells. (C) ATP-binding proteins show a trend toward increased stability in intact cells as compared with cell extract. The plots show density distributions of protein T_m values determined in intact cells or in cell extract, and the density distribution of the difference between both settings. A set of 440 proteins annotated as ATP binders show a trend toward increased stability in intact cells. The difference in the means of the distributions was significant ($P < 0.01$, t test).

changes in ligand-induced thermostability and to enable the ranking of binding affinities among multiple targets, we tailored the isothermal dose-response (ITDR) protocol to a defined set of proteins (12). In contrast to the full temperature profiles in which a compound is assayed at a single, typically saturating concentration across 10 temperatures, an ITDR profile is generated at a defined temperature, over a range of compound concentrations. To generate affinity data for GSK3182571, we acquired ITDR profiles (table S6), which showed excellent reproducibility (fig. S8) and were in good agreement with data from kinobeads competition-binding experiments, both for proteins stabilized and proteins destabilized by ligand binding (Fig. 4B and table S7).

Because of its unbiased nature, thermal proteome profiling can detect unexpected off-targets of drug compounds. The heme biosynthesis pathway has not been related to adverse drug effects but is linked to a number of genetic disorders, including erythropoietic protoporphyria, which is caused by a deficiency in FECH and results in high tissue levels of protoporphyrins (24). We found two enzymes in the heme pathway interacting with staurosporine and thus asked whether drug interactions with this pathway might result in adverse effects such as skin or liver disease (25). The melanoma drug vemurafenib frequently causes severe photosensitivity concomitant with increased levels of protoporphyrins (26). ITDR profiling of cells treated with vemurafenib showed

that the main proteins affected were its known target BRAF [negative logarithm of the median effective concentration (pEC_{50}) = 6.1] and FECH (pEC_{50} = 5.3) (Fig. 5A and table S8). These concentrations are below the typical steady-state exposure for this drug (27) and indicate a less than 10-fold window of target occupancy between the drug's efficacy target and FECH. Alectinib, a second-generation anaplastic lymphoma kinase (ALK) inhibitor for the treatment of non-small-cell lung cancer, was also reported to cause photosensitivity, in contrast to the first-generation drug crizotinib (28, 29). ITDR profiling revealed that alectinib more potently affected FECH than did vemurafenib, whereas crizotinib had no effect (Fig. 5B). These results strongly suggest that the

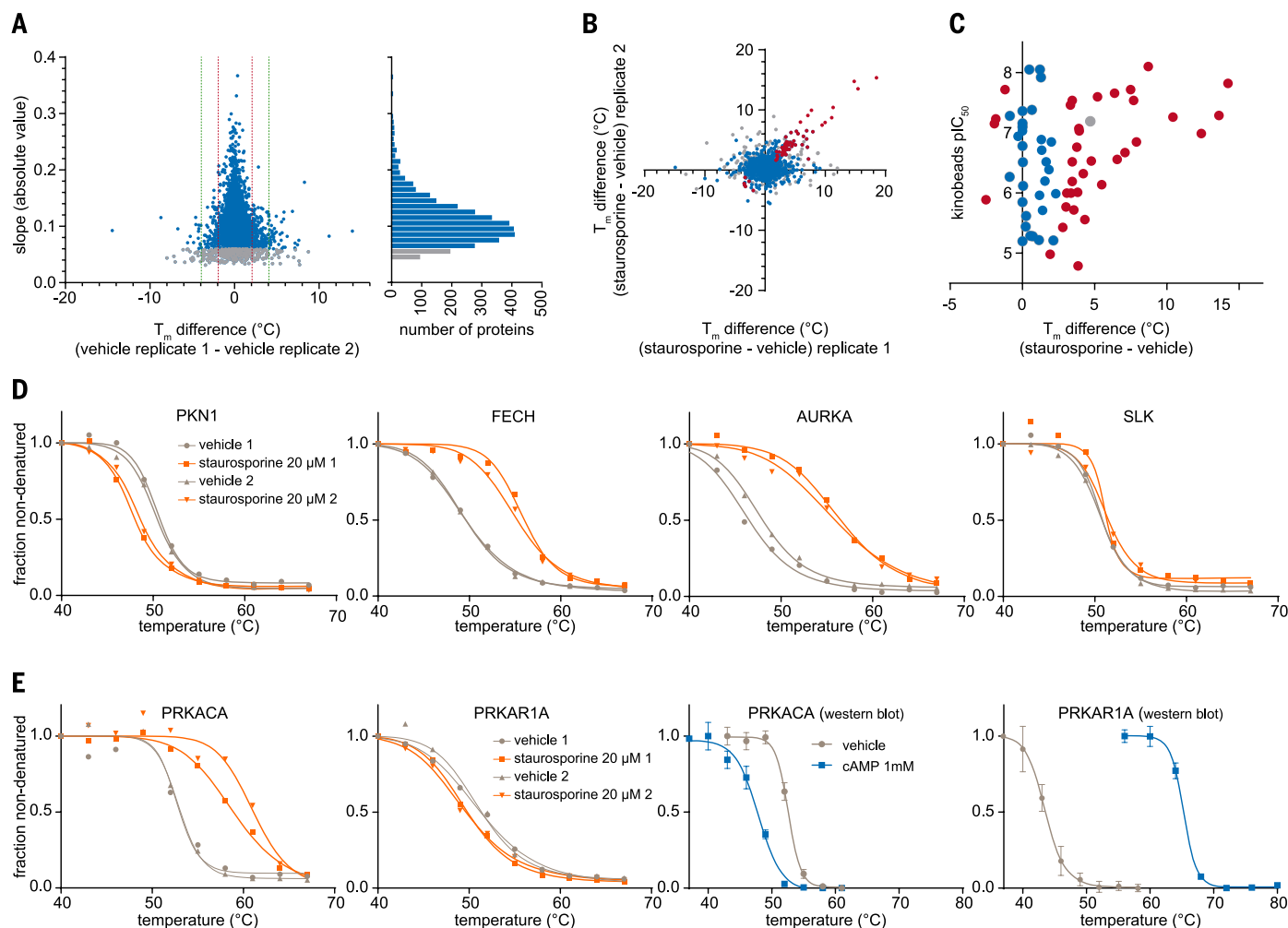


Fig. 3. Differential profiling of drug effects on the thermal proteome profile. Staurosporine treatment of cell extract yields reproducible thermal shifts, allowing robust target identification. (A) Cell extract was treated with vehicle or staurosporine. Both experiments were performed as two fully independent replicates. A flat slope of the melting curve relates to lower melting point reproducibility. Melting point differences from the two vehicle experiments are plotted against the shallowest melting curve slope observed in the four vehicle/staurosporine data sets. Proteins with an absolute slope below 0.06 are plotted in gray. The histogram shows the distribution of proteins versus slope values. Of all proteins, 92% yielded curves with sufficiently large slopes. (B) Scatter plot of T_m shifts calculated from the two

replicates of the staurosporine versus vehicle treatment experiment. Staurosporine-induced T_m shifts that passed the significance criteria are shown in red. Proteins with flat slopes—as shown in gray in (A)—are again shown in gray. (C) Comparison between T_m shifts and pIC_{50} values for staurosporine as reported from kinobeads data (22). (D) Examples of melting curves for PKN1, FECH, AURKA, and SLK with and without staurosporine treatment. (E) The catalytic subunit of PKA is stabilized by staurosporine, whereas the regulatory subunit is destabilized. Addition of cAMP followed by Western blot detection revealed destabilization of the catalytic subunit and stabilization of the regulatory subunit. Error bars indicate the SEM from $n = 4$ experiments.

photosensitivity induced by these drugs is mediated by FECH and demonstrates that thermal proteome profiling can serve as a stand-alone technique for obtaining quantitative affinity data for target-ligand interactions in a cell-based setting.

Drug treatment induces T_m shifts for downstream effector proteins

Any modification of a protein can in principle affect its thermal stability. Therefore, comparison of T_m shifts in cell extract, where ligand binding but no downstream effects occur, with T_m shifts in intact cells, where active signaling takes place, might reveal effector proteins downstream of the target. As a model system, we used K562

cells, which are transformed by an oncogenic fusion protein, the BCR-ABL kinase (30). Overall, the samples from intact cells yielded a similar protein coverage as that of samples from cell extracts (tables S9 and S10). Cultured cells were treated with vehicle or with the ABL inhibitor dasatinib, a marketed drug against chronic myelogenous leukemia (CML) (31) at two concentrations, 0.5 and 5 μM , and the samples were profiled (Fig. 6A and table S9). The minimum T_m shift for each protein was calculated from a pair of replicate experiments. Four proteins displayed significant melting point differences in both the 0.5 and 5 μM dasatinib-treated cell samples. We did not observe a T_m shift for the direct target

BCR-ABL, suggesting that this protein is not stabilized by dasatinib binding. However, substantial and significant T_m shifts were observed for the adaptor protein CRKL and the phosphatase SHIP2 (Fig. 6B and fig. S9), which is also known as INPPL1. These proteins are not likely binding the drug directly, and consistent with this, no T_m shift was observed for either protein in cell extracts (table S10). In addition, the BCR-ABL-interacting protein CRK showed a pronounced change in melting curve slope in the dasatinib-treated cells (fig. S9). CRK and CRKL are members of a proto-oncogene family that bind to tyrosine-phosphorylated proteins and are known downstream effectors of BCR-ABL signaling (32). CRKL has been previously proposed as a treatment response biomarker (33). Comparison of the CRKL melting curves in K562 cells with those in Jurkat cells (table S11), which are not transformed by tyrosine kinase signaling, revealed that dasatinib treatment of K562 cells reverted the thermal stability of CRKL to the level observed in Jurkat cells (Fig. 6C), and the same effect was observed for CRK (fig. S10). The Jurkat cell profiles also revealed that ABL1 kinase appeared to be more thermostable compared with the BCR-ABL fusion protein in K562 cells, suggesting that thermal profiling could be used to identify protein fusions which are difficult to identify by expression proteomics (Fig. 6C). In order to evaluate the drug concentration dependence of the T_m -shifted effector proteins, we performed ITDR profiles at three different temperatures (Fig. 6D, fig. S11, and table S12). The concentrations eliciting half-maximal response of the CRKL marker in the ITDR profiles recorded at three different temperatures were between 1.5 and 3.2 nM, which is in good agreement with the known potency of dasatinib for the inhibition of cell growth (34). In-depth analysis of the 50°C ITDR data revealed additional markers that either showed small T_m shifts or, in the case of the known downstream effector CRK, a pronounced change in the melting curve slope, indicating that the ITDR profiles can offer greater sensitivity than the full-temperature profiles (fig. S11). It is tempting to speculate that the most sensitive biomarkers are those that exhibit large or even stoichiometric changes in protein state, which should be robustly detected by their T_m shift. This contrasts with the use of a

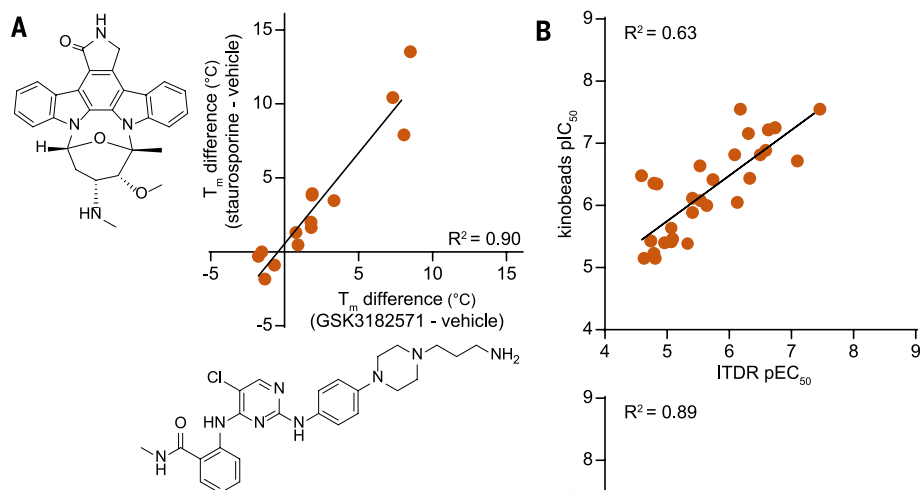
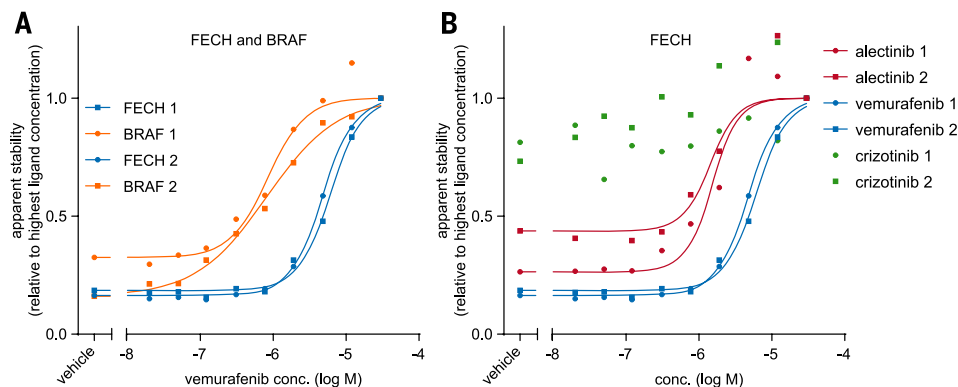


Fig. 4. Structurally unrelated kinase inhibitors with similar affinities induce T_m shifts of comparable magnitude, but assessment of ligand affinity requires ITDR measurements. (A) Staurosporine T_m shift data are compared with data for a structurally unrelated promiscuous kinase inhibitor, GSK3182571. Comparison of T_m shifts induced by staurosporine or GSK3182571 determined in cell extract. Protein targets shown had a $pIC_{50} > 6$ both for staurosporine and GSK3182571 as determined by means of kinobeads and did not differ in affinity between the two compounds by more than one order of magnitude. (B) Good agreement between GSK3182571 pEC_{50} values determined by means of ITDR, with pIC_{50} values determined with kinobeads. (Top) Proteins stabilized by GSK3182571 treatment. (Bottom) Proteins destabilized by GSK3182571 treatment.

Fig. 5. The clinical kinase drugs vemurafenib and alectinib, which can cause phototoxicity as a side effect, induce T_m shifts in the heme biosynthesis enzyme FECH.

(A) Concentration-dependent target occupancy of vemurafenib and its cognate target BRAF compared with the off-target FECH. ITDR profiling was performed at 55°C with vemurafenib-treated K562 cells and showed concentration-dependent thermal stabilization of FECH and BRAF. The data are normalized so that the quantity of soluble target at the highest compound concentration, which reflects maximum thermal stabilization, is set to 1 and fixed for curve-fitting. (B) ITDR performed at 55°C with K562 cells treated with vemurafenib, alectinib, or crizotinib over a range of concentrations. Alectinib displays a more potent effect on FECH as compared with that by vemurafenib, whereas crizotinib, a drug not known to cause photosensitivity, has no effect.



posttranslational modification as a biomarker where it is usually difficult to assess the stoichiometry of the modification.

In general terms, the thermal stability of a protein may be defined by its mutational status, posttranslational modifications, and bound ligands such as other proteins, cofactors, metabolites, or drugs. Fusion proteins, splice variants, and posttranslational modifications are typically undersampled and therefore not comprehensively detected in MS-based proteomics. Hence, the thermal proteome profile of a human cell can provide a general view of the proteomic state, or proteotype.

The future scope and applicability of thermal proteome profiling will substantially benefit from continued advances in the sensitivity and accuracy of MS-based proteomics. Future developments should include the development of protocols for membrane proteins and the application to tissues from animal studies or clinical biopsies.

Materials and methods

Reagents and cell culture

Reagents and media were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. PBS was prepared by using 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄ to buffer at pH 7.4 and supplemented with complete EDTA-free protease inhibitor cocktail (one tablet per 25 ml) (Roche Diagnostics, Basel

Switzerland). Staurosporine was from Calbiochem (Millipore, Billerica, MA), vemurafenib and crizotinib from Selleck (Houston, TX), alectinib from MedchemExpress (Monmouth Junction, NJ), and dasatinib from Toronto Research (Toronto, Canada). The synthesis of GSK3182571 is described in the supplementary methods. DNA fragments PG1 (5'-AGC TTA GAC ATG CCT AGA CAT GCC TA -3') and PG2 (5'-AGC TTA GGC ATG TCT AGG CAT GTC TA -3') were from Life Technologies (Carlsbad, CA) and dissolved in 100 mM Tris (pH7.5), 1 mM NaCl and 10 mM EDTA. K562, Jurkat E6.1, and A549 cells were from ATCC; K562 and A549 cells were cultured in RPMI medium containing 10% fetal calf serum (FCS) and Jurkat E6.1 cells in RPMI1640 supplemented with 4.5 g/l glucose, 10 mM HEPES, 1 mM sodium pyruvate, and 10% FCS. HT-29 (ATCC no. HTB-38) were maintained in Dulbecco's Modified Eagle Medium. Culture media were supplemented with 0.3 g/L L-glutamine and 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA), 100 units/mL penicillin, and 100 units/mL streptomycin. The cells were expanded to a maximum of 2×10^6 cells/ml in case of K562 and 10^7 cells/ml in case of Jurkat E6.1.

Preparation of cell extract for CETSA with Western blot read-out

A549 and HT-29 cells were harvested and washed with PBS. The cells were diluted in KB buffer

(25 mM Tris-HCl supplemented with 5 mM beta-glycerophosphate, 2 mM DTT, 0.1 mM Na₂VO₄, 10 mM MgCl₂, and complete protease inhibitor cocktail). The cell suspensions were freeze-thawed three times by using liquid nitrogen. The soluble fraction was separated from the cell debris by centrifugation at 20,000 g for 20 min at 4°C. For the CETSA melting curve experiments, cell extracts were diluted with KB buffer and divided into two aliquots, with one aliquot being treated with ligand/DNA fragments depending on the target and the other aliquot treated with the solvent of the ligand (control). After 10 min incubation at room temperature, the respective cell extracts were heated individually at different temperatures for 3 min in a thermal cycler [Applied Biosystems (Foster City, CA)/Life Technologies] followed by cooling for 3 min at room temperature. The heated cell extracts were centrifuged at 20,000 g for 20 min at 4°C in order to separate the soluble fractions from precipitates. The supernatants were transferred to new 0.2 mL microtubes and analyzed by means of Western blot analysis. NuPage Bis-Tris 4-12% polyacrylamide gels with MES SDS running buffer (Life Technologies) were used for separation of proteins in the samples. Proteins were transferred to nitrocellulose membranes by using the iBlot system (Life Technologies). Primary antibodies anti-PKA Regulatory I- α (sc-136231), anti-PKA Catalytic- α

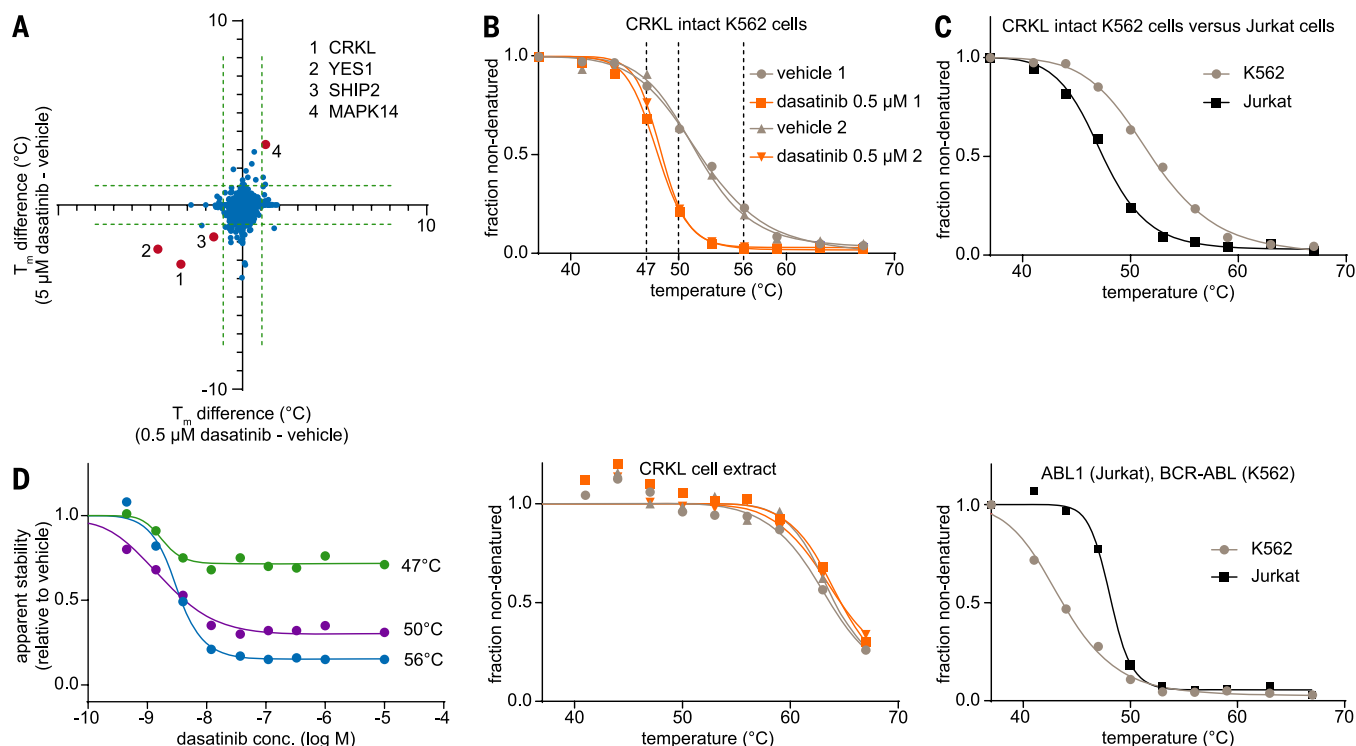


Fig. 6. Treatment of K562 cells with dasatinib induces T_m shifts for downstream effector proteins in the BCR-ABL pathway. (A) Cell-wide assessment of T_m shifts induced by dasatinib (0.5 and 5 μ M) in K562 cells. The minimum T_m shift for each protein was calculated from a pair of full replicate experiments. Four proteins showing significant melting point differences both in the 0.5 and 5 μ M dasatinib data sets are marked in red. **(B)** Effect of dasatinib on the melting curves for the effector protein CRKL,

determined in two biological replicates in intact cells (top) and cell extracts (bottom). **(C)** The melting curve for CRKL in Jurkat cells closely matches the curve obtained in dasatinib-treated K562 cells (top). The ABL1 protein in Jurkat cells is much more thermostable compared with the BCR-ABL fusion protein in K562 cells. **(D)** ITDR curves for CRKL in dasatinib-treated intact cells. Experiments were performed at 47°C (green curve), 50°C (purple curve), and 56°C (blue curve).

(sc-28315), anti-p53 WT, and R273H (sc-126); secondary goat anti-mouse HRP-IgG (sc-2055) and bovine anti-goat HRP-IgG (sc-2352) antibodies (Santa Cruz Biotechnology, Dallas, TX) were used for immunoblotting. Chemiluminescence intensities were detected and quantified by using a ChemiDoc XRS+ imaging system with Image Lab software (Bio-Rad, Hercules, CA). Data were expressed as means $\pm$ SEM. The data (band intensities) from multiple runs ($n \geq 3$) were plotted with Graphpad Prism software.

Preparation of cell extract for thermal proteome profiling

Suspension cultures of K562 cells (1.5 to 2×10^6 cells/ml) or of Jurkat E6.1 cells (4×10^6 cells/ml) were centrifuged at 340 g for 2 min at 4°C . The cells were resuspended in 50 ml PBS. After a second centrifugation step as above, the cells were resuspended in 10 ml ice-cold PBS and centrifuged again at 340 g for 2 min at 4°C . The cells were resuspended in 1.5 ml of ice-cold PBS and snap-frozen in liquid nitrogen. The tube was placed into a water bath at 23°C until $\sim 60\%$ of the content was thawed and then transferred on ice until the entire content was thawed. This freeze/thaw cycle was repeated twice before the samples were subjected to ultracentrifugation (20 min at 4°C and $100,000$ g). The protein concentration of the supernatant was determined by Bradford assay (Bio-Rad), and aliquots were snap-frozen in liquid nitrogen for use in subsequent thermal shift assays.

Thermal profiling using cell extract

A solution of the compound in dimethyl sulfoxide (DMSO) or DMSO alone as vehicle (Table 1) was added to the cell extract to 1% final DMSO concentration. The extract was then incubated for 10 min at 23°C , divided into 10 aliquots of 100 μl , and transferred into 0.2 -ml polymerase chain reaction (PCR) tubes. One each of the compound and of the vehicle containing samples was heated in parallel for 3 min to the respective temperature, followed by a 3 -min incubation time at room temperature. Subsequently, the extract was centrifuged at $100,000$ g for 20 min at 4°C . The supernatant was fractionated by means of SDS gel

electrophoresis and subjected to sample preparation for MS analysis. For ITDR experiments, GSK3182571 was tested as a nine-point serial dilution starting at 100 μM (resulting in concentrations of 100 , 33.3 , 11.1 , 3.70 , 1.235 , 0.412 , 0.137 , 0.046 , and 0.015 μM), including one vehicle control in an isothermal dose-response experiment at 53°C following the procedure outlined above for experiments in cell extract.

Thermal profiling using intact cells

To 24 ml of a suspension of K562 cells (at a density of 1.5×10^6 cells/ml) or Jurkat E6.1 cells (at a density of 4×10^6 cells/ml) in a T-flask, a volume of either 120 μl of a solution of the reference compound dissolved in DMSO or an equivalent amount of DMSO (vehicle) was added. Incubation of cells with compound and vehicle was conducted in parallel for the duration of 1 hour at 37°C and $5\%\text{CO}_2$. Cells were pelleted at 340 g and 4°C for 2 min, resuspended in 20 ml of ice cold PBS, and centrifuged again as indicated above. This washing step was repeated once, and the cells were carefully resuspended in 1200 μl PBS. 100 μl of this resulting cell suspension was transferred into 0.2 -ml PCR tubes and subjected to a centrifugation step at 325 g for 2 min at 4°C . Using a pipette, 80 μl of the PBS supernatant was removed. By gently tapping the tubes, the cells were resuspended, and one each of the compound and of the vehicle containing tubes was heated in parallel in a PCR machine for 3 min to the respective temperature (37°C to 67°C), followed by a 3 -min incubation time at room temperature. Thereafter, the cells were snap-frozen in liquid nitrogen for 1 min. The cells were thawed briefly in a water bath at 25°C , transferred on ice, and resuspended by using a pipette. This freeze-thaw cycle was repeated once. After an addition of further 30 μl of PBS and resuspension, the entire content was centrifuged at $100,000$ g for 20 min at 4°C . After centrifugation, 30 μl of the supernatant were transferred into a new tube. Protein concentration of the 37°C and 41°C samples were determined and used to normalize loading of SDS gels. Proteins in the supernatant were denatured by using SDS sample buffer, partially separated by means of SDS gel electrophoresis, and

subjected to sample preparation for MS analysis. For ITDR experiments, vemurafenib, alectinib, and crizotinib were used as a nine-point serial dilution starting at 30 μM (concentrations were 30 , 12 , 4.8 , 1.92 , 0.77 , 0.31 , 0.12 , 0.05 , and 0.02 μM), including one vehicle control at 55°C following the procedure outlined above. Dasatinib was tested as an eight-point serial dilution starting at 1 μM (concentrations used were 1 , 0.33 , 0.111 , 0.037 , 0.012 , 0.004 , 0.0013 , and 0.00046 μM), including one dasatinib control at 10 μM and one vehicle control on intact cells at 47°C , 50°C , and 56°C following the procedure outlined above for experiments on intact cells (Table 1).

Kinobeads assays

Competition binding assays were performed as described previously by using a modified bead matrix (5, 22). Briefly, 1 ml (5 mg protein) cell extract was pre-incubated with test compound or vehicle for 45 min at 4°C followed by incubation with kinobeads (35 μl beads per sample) for 1 hour at 4°C . The beads were washed with lysis buffer and eluted with 50 μl $2\times$ SDS sample buffer. GSK3182571 was tested at 10 , 2.5 , 0.625 , 0.15625 , 0.03906 , 0.00977 , and 0.00244 μM .

Sample preparation for MS

Gel lanes were cut into three slices covering the entire separation range (~ 2 cm) and subjected to in-gel digestion (5). Peptide samples were labeled with 10 -plex TMT (TMT10, Thermo Fisher Scientific, Waltham, MA) reagents, enabling relative quantification of a broad range of 10 temperature points in a single experiment (15, 22). For kinobeads and ITDR experiments, either 10 or 8 TMT labels were used depending on the compound concentration range chosen. The labeling reaction was performed in 40 mM triethylammoniumbicarbonate, pH 8.53 at 22°C and quenched with glycine. Labeled peptide extracts were combined to a single sample per experiment, and as indicated in the supplementary materials, for most samples additional fractionation was performed by using reversed-phase chromatography at pH 12 [1 mm Xbridge column (Waters, Milford, MA)], as previously described (table S13) (35).

LC-MS/MS analysis

Samples were dried in vacuo and resuspended in 0.05% trifluoroacetic acid in water. Of the sample, 50% was injected into an Ultimate3000 nanoRLSC (Dionex, Sunnyvale, CA) coupled to a Q Exactive (Thermo Fisher Scientific). Peptides were separated on custom 50 cm $\times$ 100 μM (ID) reversed-phase columns (Reprosil) at 40°C . Gradient elution was performed from 2% acetonitrile to 40% acetonitrile in 0.1% formic acid over 2 hours. Samples were online injected into Q-Exactive mass spectrometers operating with a data-dependent top 10 method. MS spectra were acquired by using $70,000$ resolution and an ion target of $3\text{E}6$. Higher energy collisional dissociation (HCD) scans were performed with 35% NCE at $35,000$ resolution (at m/z 200), and the ion target settings was set to $2\text{E}5$ so as to avoid coalescence (15). The

Table 1. Compounds used in thermal profiling. Dashes indicate that compound was not tested in intact cells.

Compound	Concentration used in cellular extract experiments	Concentration(s) in intact cell experiments	Cell line
MgATP	2 mM	—	K562
staurosporine	20 μM	—	K562
cAMP	1 mM	—	K562
GSK3182571	20 μM	—	K562
dasatinib	5 μM	0.5 μM , 5 μM	K562
p53 WT			
PG1	100 μM	—	A549
PG2	100 μM	—	A549
p53 R273H			
PG1	100 μM	—	HT-29
PG2	100 μM	—	HT-29

instruments were operated with Tune 2.2 or 2.3 and Xcalibur 2.7 or 3.0.63.

Peptide and protein identification

Mascot 2.4 (Matrix Science, Boston, MA) was used for protein identification by using a 10 parts per million mass tolerance for peptide precursors and 20 mD (HCD) mass tolerance for fragment ions. Carbamidomethylation of cysteine residues and TMT modification of lysine residues were set as fixed modifications and methionine oxidation, and N-terminal acetylation of proteins and TMT modification of peptide N-termini were set as variable modifications. The search database consisted of a customized version of the International Protein Index protein sequence database combined with a decoy version of this database created by using a script supplied by Matrix Science. Unless stated otherwise, we accepted protein identifications as follows: (i) For single-spectrum to sequence assignments, we required this assignment to be the best match and a minimum Mascot score of 31 and a 10× difference of this assignment over the next best assignment. Based on these criteria, the decoy search results indicated <1% false discovery rate (FDR). (ii) For multiple spectrum to sequence assignments and using the same parameters, the decoy search results indicate <0.1% FDR. All identified proteins were quantified; FDR for quantified proteins was <1%.

Peptide and protein quantification

Reporter ion intensities were read from raw data and multiplied with ion accumulation times (the unit is milliseconds) so as to yield a measure proportional to the number of ions; this measure is referred to as ion area (36). Spectra matching to peptides were filtered according to the following criteria: mascot ion score >15, signal-to-background of the precursor ion >4, and signal-to-interference >0.5 (37). Fold-changes were corrected for isotope purity as described and adjusted for interference caused by co-eluting nearly isobaric peaks as estimated by the signal-to-interference measure (38). Protein quantification was derived from individual spectra matching to distinct peptides by using a sum-based bootstrap algorithm; 95% confidence intervals were calculated for all protein fold-changes that were quantified with more than three spectra (36).

Curve fitting, normalization, and estimation of slope and melting point differences for CETSA experiments

Melting curves

For all CETSA experiments, fold-changes were calculated by using the lowest temperature condition as the reference. Relative fold changes as a function of temperature followed a sigmoidal trend, which was fitted with the following equation derived from chemical denaturation theory (39) (en.wikipedia.org/wiki/Equilibrium_unfolding) using R (www.r-project.org):

$$f(T) = \frac{1 - \text{plateau}}{1 + e^{-(\frac{T}{a}-b)}} + \text{plateau}$$

Where T is the temperature and a , b , and plateau are constants. The value of $f(T)$ at the lowest

temperature $T_{\min}$ was fixed to one. The melting point of a protein is defined as the temperature T_m at which half of the protein amount has been denatured:

$$f(T_m) = 0.5$$

Additionally, the slope of the melting curve is defined as the value of the first derivative of $f(T)$ at the point $T = T_{\text{inf}}$:

$$\text{slope} = f'(T_{\text{inf}})$$

where T_{inf} is the inflection point of the curve, meaning that the second derivative of $f(T)$ equals zero at $T = T_{\text{inf}}$:

$$f''(T_{\text{inf}}) = 0$$

Normalization

The vehicle and compound-treated experiments were normalized in the following way:

Selection of proteins for normalization: (i) The set of proteins, identified in both experiments termed “jointP” was determined. (ii) For each experiment (vehicle or compound) all proteins from jointP fulfilling the following criteria were collected: the fold-changes at the seventh highest temperature point compared with the lowest temperature point were between 0.4 and 0.6, and at the 9th and 10th highest temperature points compared to the lowest temperature point were below 0.3 and 0.2 respectively. (iii) The bigger of these two protein subsets was then used for normalization (typically more than 200 proteins qualified). This protein set is referred to as “normP.”

Calculation of correction factors: (i) The median fold-change values over all proteins in normP were calculated for each of the 10 fold-changes. This was done separately for both experiments, and for each experiment a melting curve was fitted. The curve that was fitted with the best R^2 was used to normalize both experiments. (ii) For each experiment, the correction factors were calculated by using the median fold-changes of the proteins in normP. For each median fold-change, a correction factor by which that fold-change would have to be multiplied with in order to coincide with the best-fitting curve was calculated.

Normalization: The two sets of correction factors were applied to all the protein fold-changes in the respective experiments.

This heuristic normalization procedure covers three important aspects. (i) The same subset of proteins is used to determine normalization coefficients in both experiments, thus circumventing any potential bias caused by proteins only identified in one experiment. (ii) The subselection of late-melting proteins (seventh point between 0.4 and 0.6) ensures that the normalization of high-temperature points will be more accurate. (iii) Last, the narrow requirements on the 7th, 9th, and 10th points will ensure that a narrow range of melting profiles is used for calculating the points from which the normalization curve will be derived. This is important because if vastly different melting profiles are combined into a single one, the above equation will not necessarily fit well. A normalization curve example as well

as melting curves of several proteins before and after normalization are illustrated in fig. S12.

Estimation of slope and melting point differences

After normalization, melting curves were fitted for all proteins identified in the vehicle- and compound-treated experiments. Melting point differences and slope differences were calculated for proteins whose curves passed the following two requirements: (i) both fitted curves for the vehicle and compound treated condition had an R^2 of greater than 0.8, and (ii) the vehicle curve had a plateau of less than 0.3.

Typically close to 80% of proteins that were identified in both the vehicle- and compound-treated experiment passed these criteria. The slope of the curves had the biggest impact on the reproducibility of melting point differences of proteins. This observation can be rationalized in the following way. If the curve has a shallow slope, then small changes with respect to the reference point—in our case, the lowest temperature—will have a strong impact on where the curve crosses the 0.5 fold-change level (melting point). Consequently, small variations in the quantitative measurement of the MS signals corresponding to the lowest temperature will lead to strong shifts in the inferred melting point of the curve. This means that it is difficult to get high precision measurements of melting point differences when comparing two curves with shallow slopes. If the curve, however, has a steep slope then the melting point measurement will not be substantially affected by small variations of the quantitative measurements of the lowest temperature, nor of any other temperature points. Taking this into account, we used the following strategy for significance estimation of protein melting point differences between vehicle- and compound-treated experiments, which is conceptually very similar to a broadly used strategy for inferring significant protein fold-changes (40). The calculated melting point differences of proteins were ordered in descending slope order (those with the shallowest slope come first), in which the slope for each protein is the lowest (steepest) slope calculated from the two curve fits performed for the protein in the vehicle- and compound-treated condition. The proteins were divided into bins. The bins are constructed starting from proteins with the highest (shallowest) slope. Each bin consists of at least 300 proteins. Once each bin has been completed, the remaining number of proteins is counted; if this number is below 300, the remaining proteins are added to the last completed bin. This data quality-dependent binning strategy is analogous to the procedure described in Cox *et al.* with the only difference being that proteins are sorted by slope instead of abundance (40). The statistical significance of differences in protein melting points was calculated by estimating the left- and right-sided robust standard deviation of the distribution of the measurements using the 15.87, 50, and 84.13 percentiles and calculating the P values for all measurements for a specific bin exactly as previously described (40). Subsequently,

an adjustment for multiple hypothesis testing was performed on the full data set by using Benjamini-Hochberg (BH) correction (41).

In order to select proteins whose thermal stability is significantly changed by compound treatment in two biological replicates (two pairs of vehicle- and compound-treated experiments) the following rules were used: (i) The melting point difference between vehicle- and compound-treated conditions for a protein had a BH-corrected P value of less than 0.05 in one biological replicate and less than 0.10 in the other. (ii) Both melting point differences were either positive or negative in the two biological replicates. (iii) The smallest absolute melting point difference of the protein in the two biological replicates was greater than the absolute melting point difference of that same protein between the two vehicle experiments. (iv) In each biological replicate, the steepest slope of the protein melting curve in the vehicle- and compound-treated conditions pair was below -0.06 .

Given the stringent requirements on the quality of the curve fits of the compared proteins, we allowed proteins quantified with single peptides to be part of the analysis.

Calculation of pEC_{50} values from ITDR experiments

For isothermal dose-response experiments, the vehicle condition was used as the reference for fold-change calculation. For proteins stabilized by the compound treatment, at least a 50% increase in protein stabilization at the highest compound concentration compared with the vehicle condition ($FC_{\text{highest concentration}} > 1.5$) was required in order to attempt to calculate a pEC_{50} . Before fitting the sigmoidal dose response curve (fitted by using GraphPad Prism, top and bottom fixed at 1 and 0 respectively, variable slope), all fold-change values were transformed as follows:

$$FC_{\text{transformed}} = (FC - 1) / (FC_{\text{highest concentration}} - 1)$$

After this transformation, the fold-change value is zero at the vehicle condition and one at the condition corresponding to the highest drug concentration. In order to ascertain that the protein stabilization was due to compound treatment and not random fluctuations, we additionally required that the fitted sigmoidal curve had an R^2 of at least 0.8. Because the transformed fold-change at the highest compound concentration condition was fixed at one, this concentration was assumed to be sufficiently high to achieve the maximum effect of the compound-induced protein stabilization.

For proteins destabilized by the compound treatment, at least a 50% increase in protein stabilization at the vehicle condition compared with the condition with the highest compound concentration was required ($FC_{\text{highest concentration}} < 0.6666$). In this case, the fold-changes were transformed as follows:

$$FC_{\text{transformed}} = (FC - FC_{\text{highest concentration}}) / (1 - FC_{\text{highest concentration}})$$

After this transformation, the fold-change value is 1 at the vehicle condition, and 0 at the con-

dition corresponding to the highest drug concentration. The sigmoidal curve fits and $R^2 > 0.8$ criterion were performed and applied in the same way as above. Conversely, here it has to be assumed that the transformed fold-change at the highest compound concentration is sufficiently high in order for the compound-induced protein destabilization to have reached the maximum effect. Given the potentially narrow fold-change range, we required proteins to be quantified with more than three spectra in order to consider them for pEC_{50} calculations.

pIC_{50} calculations

For kinobeads experiments, the vehicle condition was used as the reference for fold-change calculation. Sigmoidal dose-response curves were fitted by using R (www.r-project.org) and the drc package (www.bioassay.dk) as previously described (5).

Heat map generation

Only proteins that were quantified with at least two distinct peptides in the intact-cell experiment and additionally were quantified in the second intact-cell experiment and in the two cell-extract experiments were included (table S1). The clustering was performed in Spotfire by using complete linkage with Euclidean distance.

GO analysis

The web tool Gorilla (cbl-gorilla.cs.technion.ac.il) (42) was used to identify enriched GO terms in (i) selected clusters in the melting proteome heatmap (Fig. 2 and fig. S2) by using all the proteins in the whole-cell heatmap as background and (ii) the 200 proteins with the highest or lowest melting temperature in the whole-cells experiment and the cell extract as well as for the 200 proteins with the biggest positive or negative difference between whole cells and cell extract. For the second enrichment analysis, all proteins that were present in both the whole-cell experiment and the cell-extract experiment and fulfilled the filtering criteria described above were used as background. Significant (FDR Q value < 0.01) GO terms are reported in table S2.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/346/6205/1255784/suppl/DC1
Materials and Methods
Figs. S1 to S12
Tables S1 to S13

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RESEARCH ARTICLE

HIV EPIDEMIOLOGY

The early spread and epidemic ignition of HIV-1 in human populations

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Thirty years after the discovery of HIV-1, the early transmission, dissemination, and establishment of the virus in human populations remain unclear. Using statistical approaches applied to HIV-1 sequence data from central Africa, we show that from the 1920s Kinshasa (in what is now the Democratic Republic of Congo) was the focus of early transmission and the source of pre-1960 pandemic viruses elsewhere. Location and dating estimates were validated using the earliest HIV-1 archival sample, also from Kinshasa. The epidemic histories of HIV-1 group M and nonpandemic group O were similar until ~1960, after which group M underwent an epidemiological transition and outpaced regional population growth. Our results reconstruct the early dynamics of HIV-1 and emphasize the role of social changes and transport networks in the establishment of this virus in human populations.

AIDS is one of the most devastating infectious diseases in human history, and its cause, HIV, has been responsible for nearly 75 million infections (1). Shortly after the first reports of AIDS in the United States in 1981 (2) and the isolation of HIV-1 2 years later (3, 4), the disease was discovered to be estab-

lished in heterosexual populations of central and east Africa (5, 6), suggesting a much older—and, to that point, hidden—history of the pandemic in Africa.

Surveys of African apes identified chimpanzee [*Pan troglodytes troglodytes* (*Ptt*)] populations in southern Cameroon harboring simian immunodeficiency viruses (SIVs) most closely related to the pandemic lineage of HIV-1, group M (7, 8). HIV-1 group M comprises numerous genetically distinct virus subtypes (A, B, C, etc.) and recombinant forms. Although only group M viruses established pandemic spread, other separate cross-species transmissions of SIV to humans in the Congo River basin led to nonpandemic transmission of HIV-1 groups O, N, and P, which are still largely confined to Cameroon and its surrounding countries (9–11).

By the end of 1980s, the genetic diversity of HIV-1 group M in the Democratic Republic of Congo (DRC), then known as Zaire, was greater and more complex than that in the rest of the world (12, 13). HIV-1 strains collected in central Africa form phylogenetic outgroups to the subtypes of group M (14), suggesting that the latter are the products of incomplete sampling and exportation events (15). Two HIV-1 sequences substantially predate the discovery of AIDS and were retrospectively recovered from blood and tissue samples (16, 17) collected in Kinshasa, capital of the DRC, in 1959–1960. Other countries in the Congo River basin—notably the Republic of Congo (RC) (18, 19), as well as Cameroon and Gabon (20, 21)—also harbor very high diversities of HIV-1 comparable to that observed in the DRC. Nevertheless, hypotheses concerning the geographic

source of the pandemic and its early dissemination in humans remain controversial and have yet to be formally tested.

Although critical to our understanding of the establishment and evolution of human pathogens, a substantial period of HIV pandemic history is unclear. Despite our increased understanding of the cross-species transmissions of SIV to humans, we know very little about the early dissemination routes of HIV-1 and how group M became established as a continental epidemic in the decades immediately following its spillover from chimpanzees. Further, the genesis of major HIV-1 lineages, such as subtypes B and C, remains obscure. The lack of direct evidence about the early transmission of HIV-1 group M has led to several competing hypotheses for the emergence of AIDS (22). The two most widely accepted hypotheses for the establishment of the group M pandemic argue that urbanization and/or viral genetic factors, such as adaptation of the HIV-1 *vpu* gene (23), were decisive in the epidemiological success of group M compared with other SIV cross-species transmissions, such as group O, that did not cause pandemics.

By probing information contained in sampled viral sequences, evolutionary analyses can reveal the epidemic history of fast-evolving pathogens (24). Molecular clocks agree that a common ancestor of HIV-1 group M existed in the first half of the 20th century (16, 25–27), and models that link viral phylogenies to past transmission rates have been used to infer the epidemic history of group M (16, 27). However, several aspects of the evolutionary models used remain vulnerable to criticism (28), and the impact of recombination [a driver of HIV-1 genetic diversity (29)] on estimates of the time scale of group M spread has not been fully addressed. Using alternative methods of evolutionary analysis applied to a compilation of HIV-1 sequences from central Africa, we have uncovered the dynamics of the establishment of HIV-1 in humans, which explain how just one of many cross-species transmission events gave rise to the global pandemic we see today.

The spatiotemporal origins of pandemic HIV-1

A preliminary analysis of all available *env* C2V3 HIV-1 sequence data (30) from countries in the Congo River basin, as well as the range of *Ptt* chimpanzees, indicated that group M spread from the DRC to other countries (figs. S1 and S3); hence, we focused on this area in subsequent analyses. A very high genetic diversity of HIV-1 has been reported, not only in Kinshasa and the north and south of the DRC (12, 13, 31, 32), but also in Brazzaville in the RC and, to a lesser extent, in the Mayombe area of RC near Pointe-Noire, all of which have been suggested as potential source locations of the pandemic (22, 33, 34). We therefore performed phylogeographic analyses of viruses collected in both the DRC and RC (table S1) and compared sequence sampling locations with phylogenetic history to formally test hypotheses concerning the location of ancestral viral lineages (30). Our analyses robustly place

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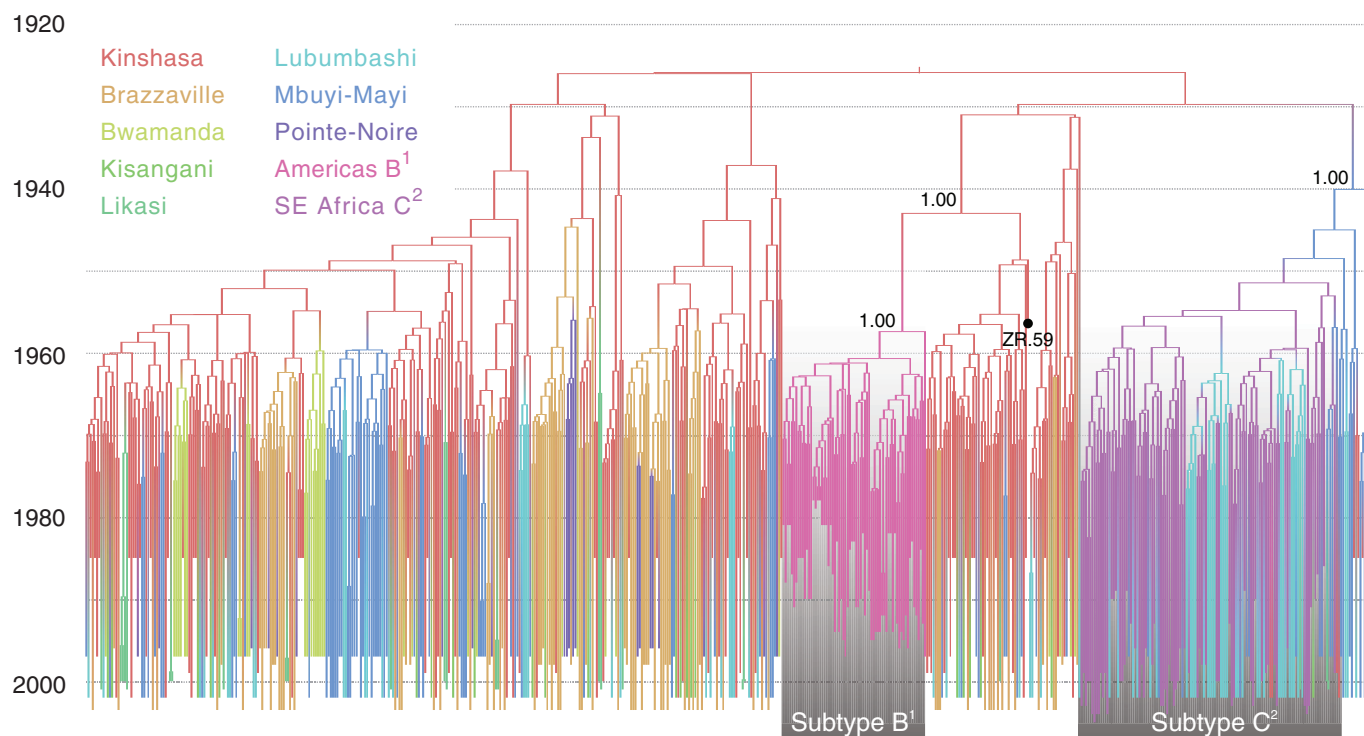


Fig. 1. Time-scaled phylogeographic history of pandemic HIV-1. Branch colors represent the most probable location of the parental node of each branch. The respective colors for each location are shown in the upper left. U.S./Haiti/Trinidad subtype B and southeast African subtype C lineages are highlighted by boxes with a gradient shading, along with the posterior probabilities for their ancestral nodes. The tip for the ZR59 sequence is highlighted with a black circle.

the spatial origin of the HIV-1 group M pandemic in Kinshasa [posterior probability (PP) = 0.99] (Figs. 1 and 2). In line with previous approaches, we estimated the time of the most recent common ancestor (TMRCA) of group M to be around 1920 [95% Bayesian credible interval (BCI): 1909–1930] (Figs. 1 and 3A). Although we focus on estimates under the best-fitting demographic model for data set A, which reduces by 39% the BCI of previous estimates (16, 25–27), the epidemic time scale we infer is robust to the evolutionary models chosen (fig. S8) and the data sets analyzed (fig. S9). Because sequence fragments for the earliest HIV-1 sample [ZR59, sampled in 1959 in Kinshasa (17)] partly overlap with the C2V3 region analyzed here, we included ZR59 as an internal control and estimated both the age and location of this strain. The estimate of the age of ZR59 is centered on 1958 (95% BCI: 1946–1970) (Fig. 3A), with little variation across data sets (fig. S9). In Fig. 3A, the posterior probability distribution of this age estimate is stratified according to the estimated location of ZR59; crucially, Kinshasa receives the highest support as the estimated location (PP = 0.81). The decisive support for Kinshasa as the epicenter of pandemic group M is robust to differences in spatial model specification and sampling heterogeneity (30) (tables S2 to S5 and fig. S4). To further test robustness, we deliberately excluded Kinshasa sequences sampled at the earliest time point (1985, representing 51% of strains for this location), which resulted in a root location at Brazzaville (PP = 0.97), located just 6 km from Kinshasa across the Congo River.

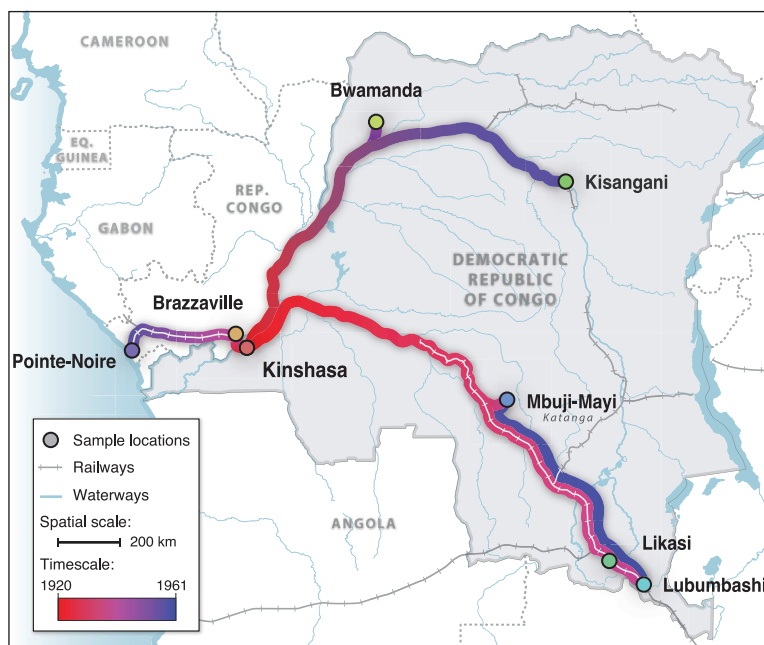


Fig. 2. Spatial dynamics of HIV-1 group M spread. Circles represent sampled locations and are colored according to the estimated time of introduction of HIV-1 group M from Kinshasa. Strongly supported rates of virus spatial movement (table S6) are projected along the transportation network for the DRC (railways and waterways), which was fully operational until 1960 (38). Gradient colors depict the time scale of spatial movements (bottom left).

Our estimated location of pandemic origin explains the observation that Kinshasa exhibits more contemporary HIV-1 genetic diversity than anywhere else (12, 13). It clarifies why the oldest

known HIV-1 sequences were sourced from this city (16, 17) and why several early cases indicative of AIDS are linked to Kinshasa (35). The cross-species transmission of SIV to humans predates

the group M common ancestor (36) and probably occurred in southeast Cameroon, where the chimpanzees with SIVcpz strains most similar to group M have been identified (7, 8). After localized transmission, presumably resulting from the hunting of primates, the virus probably traveled via ferry along the Sangha River system to Kinshasa (37). During the period of German colonization of Cameroon (1884–1916), fluvial connections between southern Cameroon and Kinshasa were frequent due to the exploitation of rubber and ivory (36).

Early spatial expansion from Kinshasa

With the geographic origins of pandemic group M clear, we next sought to investigate its spread from Kinshasa to the rest of Africa. To identify statistically significant epidemiological links among

locations and quantify virus exchange, we estimated rates of viral lineage migration using an established “robust counting” approach (30). In addition to identifying Kinshasa as the location of the group M common ancestor, our analyses showed a dynamic pattern of HIV-1 movement in the DRC and RC, dominated initially by viral dispersal away from Kinshasa and toward other population centers (Fig. 2). Overall, 57% (95% BCI: 48 to 65%) of all viral lineage movements originated from Kinshasa. Of these, one-third were directed to the neighboring city of Brazzaville (fig. S5), explaining the high genetic diversity of group M reported there (18, 19). Further, our results revealed that the earliest introductions of HIV-1 to Brazzaville occurred by 1937 (95% BCI: 1920–1953) (Fig. 3B). We note that these estimates pertain to viral lineages that survived to be sampled in each

location; thus, HIV-1 may have been introduced earlier (e.g., to Brazzaville) but without successful onward transmission. Historical transportation data from the DRC during 1900–1960 (38) (Fig. 3C) suggests that viral lineages in migrant populations living in or around Kinshasa would have had many opportunities for introduction to DRC regions connected to other population centers in central Africa (39).

Our genetic analyses indicated that the virus reached the southern DRC locations Lubumbashi and Mbuji-Mayi by ~1937 (95% BCI: 1919–1957) and ~1939 (95% BCI: 1922–1954), respectively (Fig. 3B). These two locations received ~41% of viral lineage export from Kinshasa (fig. S5). Even if we consider our most conservative dating estimates, our results indicate that group M viruses were circulating in Brazzaville and southern

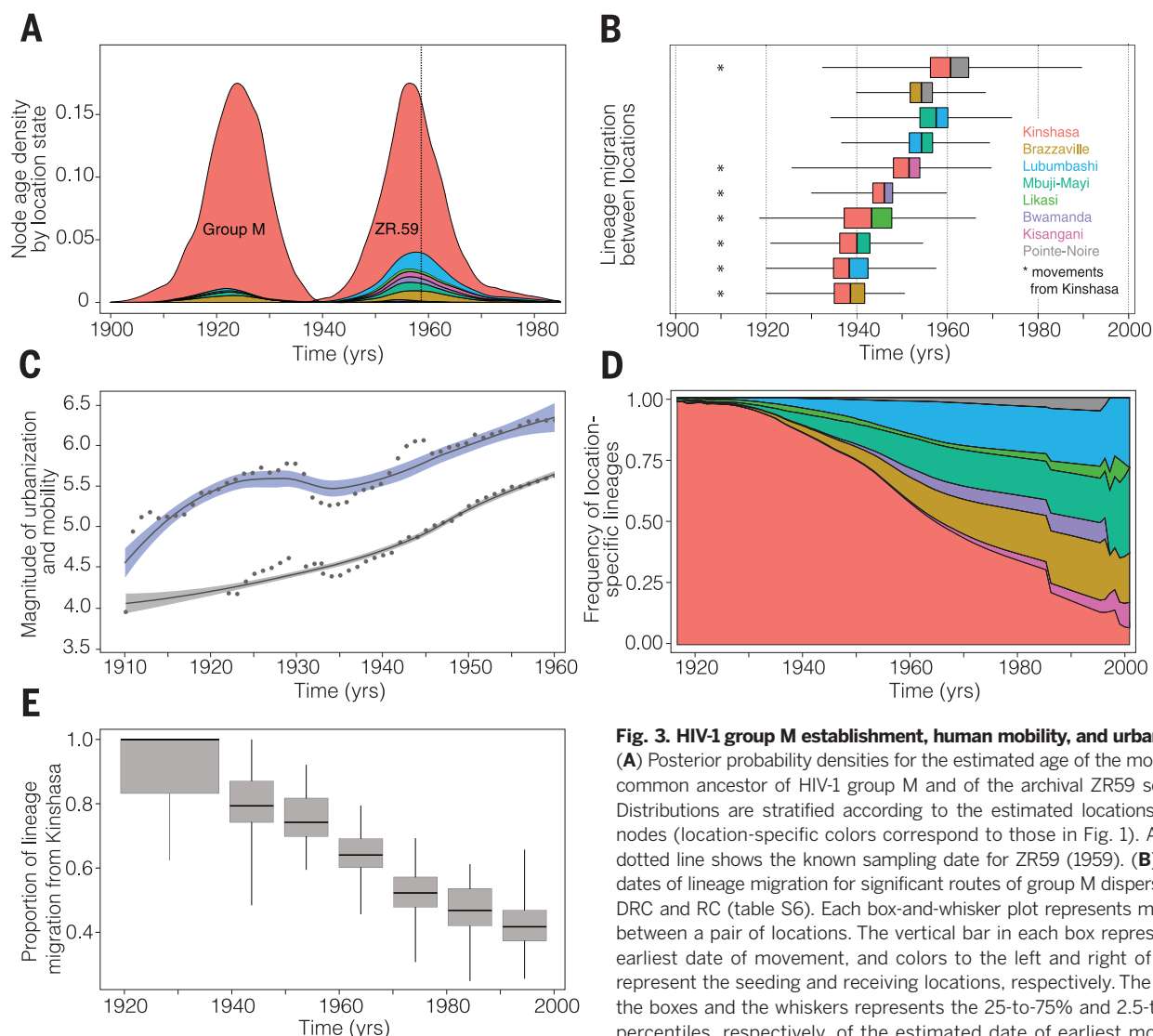


Fig. 3. HIV-1 group M establishment, human mobility, and urbanization.

(A) Posterior probability densities for the estimated age of the most recent common ancestor of HIV-1 group M and of the archival ZR59 sequence. Distributions are stratified according to the estimated locations of both nodes (location-specific colors correspond to those in Fig. 1). A vertical dotted line shows the known sampling date for ZR59 (1959). (B) Earliest dates of lineage migration for significant routes of group M dispersal in the DRC and RC (table S6). Each box-and-whisker plot represents movement between a pair of locations. The vertical bar in each box represents the earliest date of movement, and colors to the left and right of this bar represent the seeding and receiving locations, respectively. The width of the boxes and the whiskers represents the 25-to-75% and 2.5-to-97.5% percentiles, respectively, of the estimated date of earliest movement. (C) Locally weighted regression curves for the official total number of

passengers (log10) transported along railways (95% of journeys) and waterways (5%) in the DRC (38) (blue) and for the human population size (log10) of Kinshasa (gray) between 1900 and 1960 (43), after which reliable transportation data are unavailable. Dots show regression data points. (D) Estimated frequency of group M lineages at each location in the DRC and RC through time. (E) Estimated proportion of all migration events that began in Kinshasa until 1940 and, per decade, between 1940 and 2000. [Box-and-whisker widths are defined in (B).] This percentage drops to 43.5% between 1990 and 2000 (fig. S6 shows the estimated proportion of movement events originating from each location).

DRC before the date of the earliest known HIV-1 samples (1959–1960), and therefore, similar samples may exist in historical collections in locations outside Kinshasa. However, it took another decade for pandemic HIV-1 strains to seed central and northern DRC locations, reaching Bwamanda by 1946 (95% BCI: 1929–1959) (Fig. 3C) and Kisangani by 1953 (95% BCI: 1926–1970). The comparatively late arrival of pandemic HIV-1 in northeastern DRC is consistent with historical records indicating that only 5% of human journeys within the DRC occurred on the fluvial network connecting Kinshasa and Kisangani (38).

Group M arrived first at the three largest population centers—Brazzaville, Lubumbashi, and Mbuji-Mayi (40, 41)—that were better connected to Kinshasa (38), indicating a critical role for mobility networks in the early spread and establishment of HIV-1 from its epicenter (42). Within the DRC, the majority of journeys took place along the railway network, which was used by >300,000 passengers per year in 1922, peaking at >1 million annual passengers in 1948 (Fig. 3C). Mbuji-Mayi, the world's second largest producer of industrial diamonds, and Lubumbashi, also a mining city and the second largest of the DRC, were connected via the most active section of the DRC railway network (38). Although most viral movement consisted of lineage dissemination away from Kinshasa (Fig. 3D), we also identified one instance of significant bidirectional virus exchange, between Mbuji-Mayi and Lubumbashi (table S6), with the two earliest migrations between them dating back to 1957 (95% BCI: 1934–1974) and 1954 (95% BCI: 1936–1968), respectively (Fig. 3B). To further quantify changes in HIV-1 dissemination through time, we estimated, for each decade, the relative proportion of viral lineage movements that began in Kinshasa. We found a significant

decline (8% per year) in this measure (Fig. 3, D and E). By the mid 1980s, approximately half of all dispersal events were seeded from secondary locations (Fig. 3E), thereby establishing the geographically heterogeneous distribution of HIV subtypes observed across eastern and southern Africa (39).

Divergent epidemic dynamics of HIV-1 groups M and O

Whereas our data show that HIV-1 group M was already established in several DRC locations before 1960, group O remained nonpandemic and largely confined to west-central Africa. To investigate how the spatial expansion of HIV-1 in the DRC relates to its epidemic history, we estimated past growth rates for HIV-1 groups M and O in central Africa using methods based on coalescent theory (30), a population genetic model that links phylogenetic tree shape to the demographic history of the sampled population (24).

Our analyses provide an estimate of the effective number of HIV-1 infections through time for HIV-1 groups M and O. Between 1920 and 1960, group M underwent an early phase of relatively slow exponential growth (Fig. 4). Using a two-phase exponential-logistic model of population growth (30), we estimate the exponential growth rate of group M during the early phase to be 0.1 year^{-1} (95% BCI: 0.064 to 0.15 year^{-1}), close to the population growth rate of Kinshasa (0.081 year^{-1} , SD: 0.00077) (Fig. 4) (43). HIV-1 was largely restricted to Kinshasa for most of this period (Figs. 1 and 3D). For group O, we estimate slightly slower exponential growth rates of 0.071 year^{-1} (95% BCI: 0.046 to 0.099 year^{-1}), which may reflect lower infectivity caused by the greater susceptibility of group O to the antiviral host protein

tetherin (23). This suggests that genetic factors specific to the SIV ancestors of HIV-1 that infected chimpanzees and gorillas may have been most important in the period immediately after cross-species transmission.

However, around 1960 (95% BCI: 1952–1968) group M transitioned to a second, faster phase of exponential growth (Fig. 4; see also fig. S8, which demonstrates robustness of the estimated growth parameters to the molecular clock model used). During this second period, group M growth rates more than doubled to 0.27 year^{-1} (95% BCI: 0.20 to 0.33 year^{-1}), substantially outpacing the concurrent rate of Kinshasa population growth. Our results thus call into question the role of human population expansion in HIV-1 emergence (33). Crucially, the estimated time of this transition also marks the time at which the epidemic histories of groups M and O diverge. Although the TMRCA of group O (1926; 95% BCI: 1903–1948) is similar to that of group M and both grew at similar rates until ~1960, group O exhibits no subsequent increase in growth rate and remains largely confined to Cameroon and surrounding countries (10). Whereas virus-specific factors may explain the differences in early-phase growth rates, invoking this hypothesis to explain the group M transition between 1952 and 1968 would require the implausible proposition that viral accessory genes evolved concurrently and convergently in multiple lineages already present in different central African locations (Figs. 1 and 3B and fig. S3). Lastly, Fig. 4 indicates a stabilization in epidemic growth over the past two decades. Although the methods used here may sometimes underestimate growth rates near the present (44), this slowdown agrees with reports of relatively stable HIV prevalence in the DRC from 1976 to 1997 (45, 46).

The observation that HIV-1 group M growth rates nearly tripled around 1960 is a consequence of a relative increase (at that time) in the rate at which sampled viral lineages join together, or coalesce, as time proceeds backward toward the phylogeny root. Theory suggests three non-mutually exclusive explanations for this change in the coalescence rate. (i) Group M viruses expanded geographically and established new subpopulations around 1960 (47), resulting from the dispersal of sampled lineages from Kinshasa (Fig. 3). (ii) Group M transmission rates increased, in Kinshasa or elsewhere, such that the number of infections was substantially lower before ~1960. (iii) Onward transmission per capita was more homogeneous and, on average, less frequent after the estimated transition (95% BCI: 1952–1968). This counterintuitive result arises because the lineage coalescence rate will be faster when only a small fraction of infections generate the majority of new cases and when the viral generation time is reduced (48). To discriminate among these hypotheses, we first reconstructed the epidemic history of lineages that maintained ancestry within Kinshasa (i.e., 84 taxa in Fig. 1 that have exclusively red branches in their ancestry that were sampled between 1985 and 2002; PP cut-off > 0.80). Because this procedure recovers a similar

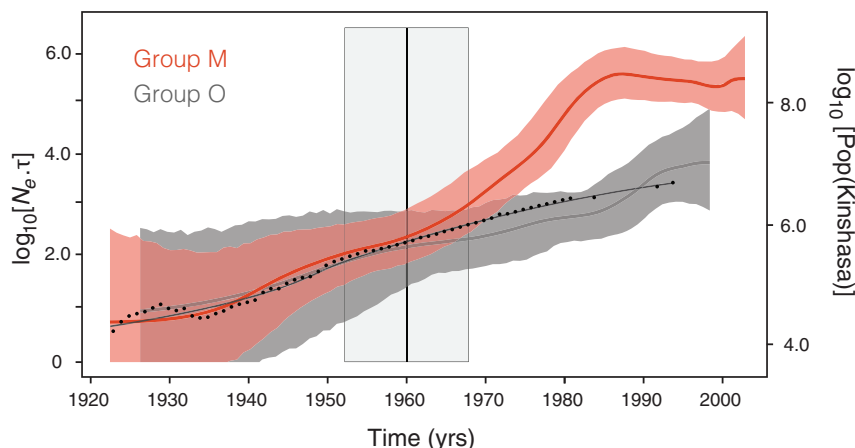


Fig. 4. Population dynamics of HIV-1 groups M and O. Bayesian skygrid estimates of past population dynamics for group M (red) and group O (gray) (30). The left y axis represents the effective number of infections (N_e) multiplied by the mean viral generation time (τ). Group O dynamics were obtained using the same best-fitting demographic model as for group M (table S7), applied to an alignment of 50 concatenated *gag*, *pol*, and *env* sequences sampled between 1987 and 1999 from west-central African patients (62). The superimposed black curve represents a locally weighted regression of human population growth in Kinshasa between 1920 and 1994 (43, 64). Dots show regression data points. The vertical line at 1960 corresponds to the estimated time at which group M transitioned from slow to faster exponential growth. The 95% BCI for this estimate is highlighted in gray.

epidemic profile (fig. S7) to that in Fig. 4, it seems unlikely that geographic expansion directly drove the change in coalescence rate, despite it being a necessary condition for the international establishment of the pandemic.

Explanations (ii) and (iii) are compatible with an early establishment of group M in high-risk groups of small size—for example, commercial sex workers with higher rates of partner exchange and/or exposure to contaminated injections—before later spreading to the larger, general DRC population from the 1950s onward. Specifically, the transition to faster exponential growth (Fig. 4) agrees with available public health data (34) and the hypothesis that transmission rates of group M increased as a result of the administration of unsterilized injections at sexually transmitted disease clinics in the 1950s and/or subsequent changes in the nature of commercial sex work in Kinshasa from the early 1960s, which led to increased client numbers (34). The idea that early HIV spread included an iatrogenic component is supported by data from other blood-borne viruses. A study of hepatitis C virus (HCV) in the DRC showed that it exhibits an age cohort effect (49), and an epidemic of hepatitis [presumably hepatitis B virus (HBV)] was reported in Kinshasa in 1951–1952 (50). Both events indicate an important role for past iatrogenic transmission. Additional genetic data may allow the past dynamics of HCV, HBV, and other viruses in the DRC to also be reconstructed.

It seems less likely that genital ulcer disease (GUD) or circumcision practices played a role in the group M transition. In 1920, GUD incidence was ~10% for primary and secondary syphilis and chancroid but dropped by a magnitude of 1.5 to 2.5 until 1960 (51). It is conceivable that post-independence changes in sexual behavior could have increased GUD incidence, but unfortunately this is difficult to assess, as postcolonial medical records are scarce or nonexistent. Further, a lack of circumcision was unlikely to have played a role, as nearly all males in Kinshasa were circumcised by 1960 (51).

The emergence of HIV-1 subtypes

Unlike HIV-1 strains from outside Africa, group M viruses from the DRC are not structured into clearly distinct subtypes (14). The former are exemplified by subtype B, which forms a distinct monophyletic cluster within the group M phylogeny (Fig. 1). It is thought that subtype B originated as a viral lineage exported from Africa to Haiti (52) and then to the United States, from where it spread internationally to become the most geographically dispersed subtype worldwide (53). Our analyses indicate that the lineage ancestral to subtype B originated in Kinshasa ($PP = 0.99$) (Fig. 1). This lineage was already present in Kinshasa by 1944 (95% BCI: 1935–1951) and, in agreement with previous findings (52), arrived in Haiti around 1964 (95% BCI: 1960–1967). It has been suggested this occurred with the return of Haitian professionals who worked in the newly independent Congo in the 1960s (54, 55). Our results strengthen this hypothesis, as a large

proportion of these professionals were based in Kinshasa (55, 56).

In contrast to subtype B, subtype C spread successfully within Africa and currently accounts for ~50% of HIV-1 infections worldwide (53). Our phylogeographic reconstruction suggests Mbuji-Mayi as the most likely ancestral location of subtype C ($PP = 0.56$) (Fig. 1). Moreover, south and east African subtype C sequences are phylogenetically interspersed with sequences from Lubumbashi, capital of the southern Katanga province. Therefore genetic and historical data indicate independently that the DRC transportation network provided the key connection between the Kinshasa region and other human population centers in sub-Saharan Africa (Figs. 2 and 3 and table S6), and additionally provided a link between southern DRC and neighboring Zambia and Angola (38). This indicates subtype C as a lineage that developed in the DRC mining regions, from where it spread south and east, probably through migrant labor. The impact of migrant labor on the spread of HIV-1 is well established in southern Africa (57), where subtype C dominates with high prevalences (53).

Impact of recombination and evolutionary rate heterogeneity

The cocirculation of divergent HIV-1 subtypes has facilitated the identification of recombinant HIV lineages (29). Recombination may confound phylogenetic reconstructions and may adversely affect molecular clock estimates (58, 59). Although we perform evolutionary reconstructions on relatively short sequence fragments (averaging 391 nucleotides) with limited opportunity to contain recombination breakpoints (60), we also conducted extensive simulations to assess the potential effect of recombination on the estimation of divergence times, evolutionary rates, and viral growth rates (30). These analyses confirm that recombination does not significantly affect our TMRCA estimation. Even for rates of 3×10^{-4} recombinations per site per year [about one order of magnitude higher than rates reported for group M (60, 61)], the variance of the TMRCA of group M increased only by 5.3% (tables S8 and S9). Thus, even for levels of recombination that are much higher than expected, the potential bias on key parameters inferred here is limited (62).

Evolutionary rates may also vary among subtypes, and it has been suggested that relaxed molecular clock models may have difficulties accommodating this rate heterogeneity (63). We addressed this by investigating the robustness of our estimates with respect to the inclusion of subtype B and C sequences. Although evolutionary rate estimates for data sets comprising only subtype B or C result in slightly slower rates (2.90×10^{-3} and 2.47×10^{-3} substitutions per site per year, respectively) than those estimated for the complete group M data set (3.26×10^{-3} substitutions per site per year), analyses of group M divergence times without subtypes B and C produce a similar TMRCA estimate (1926; 95% BCI:

1918–1934), indicating that evolutionary rate variation is accommodated satisfactorily here by a relaxed clock model (fig. S9).

Conclusions

We show that the HIV-1 group M pandemic ignited in Kinshasa around the early 1920s and that its spatial expansion in central Africa was contingent upon an active transportation network that connected the country's main population centers to other regions of sub-Saharan Africa. Further, the increase in the exponential growth rate of group M around 1960 stands in contrast to that of the spatially confined, non-pandemic group O. Our results are consistent with hypotheses that iatrogenic interventions in Kinshasa and its surroundings and/or post-independence changes in sexual behavior were critical for the emergence of group M (22). We suggest that a distinct combination of circumstances during a particular spatial and socio-historical window permitted the establishment, spatial dissemination, and epidemic growth of the HIV-1 group M pandemic. Similar arguments may underlie the emergence of other blood-borne pathogens, particularly that of HCV.

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the experiments and designed the study. N.R.F. and P.L. conducted the phylogenetic analyses. D.P. performed the recombination analysis and G.B. the model selection analysis. M.A.S. and T.B. contributed methodology. T.B., M.J.W., and N.A. assisted the sequence analysis. J.P., A.J.T., and J.D.S. contributed historical and spatial data. M.P. provided sequence data. N.R.F., P.L., O.G.P., and A.R. wrote the paper. All authors discussed the results and approved the final manuscript. We declare no competing financial interests.

SUPPLEMENTARY MATERIALS

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REPORTS

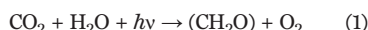
PHOTOCHEMISTRY

Evidence for direct molecular oxygen production in CO₂ photodissociation

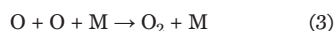
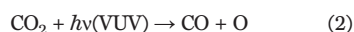
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Photodissociation of carbon dioxide (CO₂) has long been assumed to proceed exclusively to carbon monoxide (CO) and oxygen atom (O) primary products. However, recent theoretical calculations suggested that an exit channel to produce C + O₂ should also be energetically accessible. Here we report the direct experimental evidence for the C + O₂ channel in CO₂ photodissociation near the energetic threshold of the C(³P) + O₂(X³Σ_g[−]) channel with a yield of 5 ± 2% using vacuum ultraviolet laser pump-probe spectroscopy and velocity-map imaging detection of the C(³P_j) product between 101.5 and 107.2 nanometers. Our results may have implications for nonbiological oxygen production in CO₂-heavy atmospheres.

It is widely accepted that the rise of the oxygen-rich atmosphere on Earth, known as the “Great Oxidation Event,” occurred at ~2.4 billion years ago via multistep photosynthetic processes (Eq. 1) (1, 2)



Here, h is Planck's constant and ν is the frequency. Over the past 40 years, biologists and paleontologists have proposed that free oxygen molecules must have been available in small quantities before the rise of oxygenic photosynthesis in Earth's prebiotic primitive atmosphere (3). The only known abiotic production mechanism was through solar vacuum ultraviolet (VUV) photodissociation of CO₂ to form CO + O in the early Earth stratosphere, followed by the three-body recombination reactions shown in Eqs. 2 and 3



Here, M is a third body for carrying off the excess energy involved in the formation of the O₂ chemical bond (4–6). Decades of experimental and theoretical photochemical studies of CO₂

have been focused on the detection and understanding of the CO + O photoproduct channels.

Recent theoretical calculations (7, 8) suggest that an exit channel to produce C + O₂ upon VUV photoexcitation of the CO₂ molecule is possible. The ab initio calculation (7) has provided the dissociation pathway on the ground-state singlet potential energy surface of CO₂, leading to the formation of the C(³P) + O₂(X³Σ_g[−]) products (where X is indicative of the ground state) (pathway 1 of Fig. 1). If the electronically excited singlet CO₂ molecule initially produced by photoexcitation undergoes internal conversion to the ground-state singlet potential surface, the O atom could migrate through a cyclic CO₂ complex [c-CO₂(¹A₁)] and form a colinear COO(¹Σ⁺) intermediate before dissociation to C(³P) + O₂(X³Σ_g[−]), as shown in pathway 1. The theoretical calculation (7) predicts no potential energy barrier for this dissociation pathway. Grebenshchikov (8) calculated the singlet ground and excited potential energy surfaces of COO, with the O-O bond distance fixed at 2.3 bohr. His calculations indicate that the singlet ground and excited surfaces are connected by conical intersections, and his results support Hwang and Mebel's conclusion (7) that there is no potential energy barrier via the COO colinear state to yield C(³P) + O₂(X³Σ_g[−]) on the ground-state singlet surface. Despite these theoretical results, to our knowledge there has been no experimental verification of the C + O₂ channel in CO₂ photodissociation. Here we present the experimental evidence of C(³P) + O₂(X³Σ_g[−])

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products based on VUV-pump and VUV-probe velocity-map imaging (VMI) measurements of $C(^3P_2)$ atoms. Direct detection of $O_2(X^3\Sigma_g^-)$ photoproducts is not feasible, in part due to the substantial number of O_2^+ background ions produced by photoionization of ambient O_2 molecules in the experimental chamber. Furthermore, state-selected VUV photoionization of $O_2(X^3\Sigma_g^-)$ photofragments would result in a much lower signal intensity compared with that of $C(^3P_1)$ atoms because a small fraction of $O_2(X^3\Sigma_g^-)$ products at a single rovibrational state is detected among the nascent $O_2(X^3\Sigma_g^-)$ produced in a wide range of rovibrational states, whereas the $C(^3P_1)$ atoms are only formed in three spin-orbit states.

In this CO_2 photodissociation experiment, the VUV photoexcitation energy $h\nu(VUV)$ is distributed between the $C(^3P_1)$ and $O_2(X^3\Sigma_g^-)$ product translational energy (E_{trans}) and internal energy (E_{int}), where $E_{int}[C(^3P_1)]$ is due to excitation of the spin-orbit state; and the correlated $O_2(X^3\Sigma_g^-)$ photoproducts can be both rotationally and vibrationally excited. The VMI technique can be used to determine the recoil velocity distribution of the $C(^3P_1)$ photofragments from the radii of the resolved ring structures in the velocity-map image. The velocities are converted to a total kinetic energy release (TKER) spectrum of the $C(^3P_1) + O_2(X^3\Sigma_g^-)$ channel by using Eq. 4 based on the conservation of linear momentum and energy

$$h\nu(VUV) = D_0 + E_{int}[C(^3P_1)] + E_{int}[O_2(X^3\Sigma_g^-)] + E_{trans}[C(^3P_1) + O_2(X^3\Sigma_g^-)] \quad (4)$$

Here, $D_0 = 11.44$ eV is the thermochemical threshold for the formation of the $C(^3P_1) + O_2(X^3\Sigma_g^-)$ products from CO_2 dissociation (7). Equation 4 can also be used to determine the internal rotational and vibrational energy populations of $O_2(X^3\Sigma_g^-)$ photofragments from the TKER spectrum.

In the present experiment, a skimmed and pulsed supersonic molecular beam generated from ~10% CO_2 seeded in He was irradiated by two counter-propagating unfocused VUV laser beams, hereafter designated as “VUV₁” and “VUV₂” (9, 10). The VUV₁ laser output was used to directly excite CO_2 to a dissociative rovibronic state. $C(^3P_1)$ photofragments were then selectively ionized via a VUV₂-visible (1+1') resonance-enhanced photoionization scheme, which has been shown to achieve substantially higher detection sensitivities for $C(^3P_1)$ detection compared with those observed using a direct VUV photoionization method (9).

The ion species formed by photoionization in the interaction region were accelerated by the ion imaging optics into a time-of-flight (TOF) spectrometer for mass analyses and detected by a dual microchannel plates detector. Figure 2 depicts three TOF spectra in the mass range of 10 to 20 atomic mass units (amu) observed in CO_2 photodissociation at $h\nu(VUV) = 11.832$ eV (95,430.0 cm^{-1}) under different photoexcitation conditions. The top red trace shows the C^+ ion peak from $C(^3P_2)$ observed at 12 amu when both VUV₁ and VUV₂ were turned on in the interaction region. No C^+ ion was observed upon tuning of the VUV₂ laser

off-resonance (black trace) or blocking of the VUV₁ beam in the interaction region (blue trace). The data thus clearly demonstrate that the observed C^+ ion intensity depends on both the VUV₁ photoexcitation of CO_2 and the VUV₂ photoionization of $C(^3P_2)$ photofragments. The ion peak at 18 amu, which was observed in all three mass spectra, is attributed to background H_2O^+ ions produced by VUV photoionization of ambient H_2O vapor in the photoionization chamber.

In Fig. 3, panels A to C compare the CO_2 photoabsorption spectrum reported by Archer *et al.* (11) to the photofragment excitation spectra obtained by detecting the $C(^3P_2)$ or the $O(^1S)$ fragments while tuning the VUV₁ over the energy range 11.562 to 12.212 eV (93,250 to 98,500 cm^{-1}). The photoabsorption spectrum of Fig. 3A shows the vibrational progressions of the $3p^1\Pi_u$ and $4s$ Rydberg states, which were identified previously by Cossart-Magos *et al.* (12) and Kuo *et al.* (13). The $C(^3P_2)$ and $O(^1S)$ photofragments were detected by VUV₂-visible (1+1') photoionization and VUV₂ excited autoionizing Rydberg state schemes with the excited $C^*[2s^22p3d(^3D^o_3)]$ and $O^*[2s^22p^3(^4P^o)3s(^1P^o_1)]$ Rydberg states, respectively, as VUV₂ resonant intermediate states. As shown in Fig. 3, A to C, the photofragment excitation spectra of $C(^3P_2)$ and $O(^1S)$ are in excellent agreement with the CO_2 photoabsorption spectrum, except that the $4s$ Rydberg peak of CO_2 at 11.967 eV (96,522 cm^{-1}) resolved in the spectrum of Fig. 3A is strongly perturbed or distorted in the $C(^3P_2)$ and $O(^1S)$ photofragment excitation spectra due to a strong dip in the VUV₁ tuning curve in this energy region. Because the $O(^1S) + CO(X^1\Sigma^+)$ channel is

known to be a major nascent photoproduct channel in CO_2 photodissociation in this VUV energy range, the excellent agreement between the photofragment excitation spectra of $C(^3P_2)$ and $O(^1S)$ and the photoabsorption spectrum of CO_2 indicates that the $C(^3P_2)$ fragments are also nascent photoproducts in CO_2 photodissociation. The identification of $C(^3P_2)$ as a nascent photofragment clearly shows that the correlated O_2 photoproduct is formed in the VUV photodissociation of CO_2 . The current photodissociation measurements on CO_2 were performed under molecular beam conditions to ensure that secondary collisions were unimportant. As pointed out above, the direct detection of $C(^3P_2)$ photofragments allows the measurement of the E_{int} distribution of the O_2 coincident photofragments using the VMI method. Because the photofragment excitation spectra of Fig. 3, B and C, have been normalized by the photodissociation VUV₁ laser intensity, which was monitored by measuring the photoionization efficiency spectrum of acetylene (C_2H_2) (14), the relative intensities of the $C(^3P_2)$ and $O(^1S)$ photofragment excitation spectra can be used to determine the relative yields of the $C(^3P_2) + O_2(X^3\Sigma_g^-)$ and $O(^1S) + CO(X^1\Sigma^+)$ photodissociation channels, respectively, in the CO_2 photodissociation energy range of $h\nu(VUV) = 11.562$ to 12.212 eV. Previous experimental studies suggest that the yield of the $O(^1S) + CO(X^1\Sigma^+)$ channel increases as the VUV photodissociation energy decreases (15), which is in good agreement with the CO_2 absorption spectrum and the observed $O(^1S)$ photofragment excitation spectrum of Fig. 3C. In comparison with the CO_2 absorption spectrum,

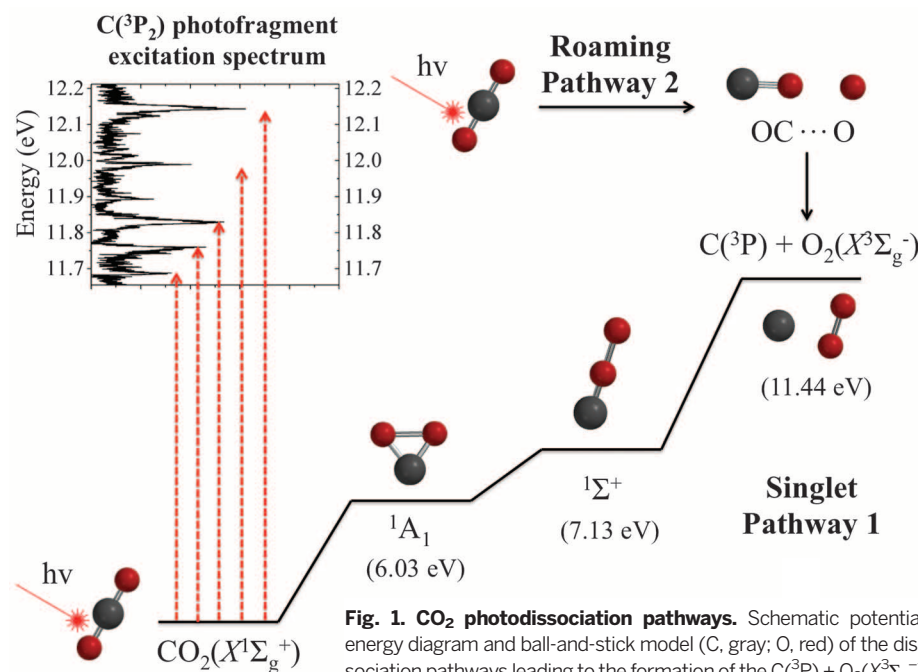


Fig. 1. CO_2 photodissociation pathways. Schematic potential energy diagram and ball-and-stick model (C, gray; O, red) of the dissociation pathways leading to the formation of the $C(^3P) + O_2(X^3\Sigma_g^-)$ products. The singlet potential energy pathway (pathway 1) is predicted to involve the formation of the $c-CO_2(^1A_1)$ and $COO(^1\Sigma^+)$ intermediates situated at 6.03 and 7.13 eV above the $CO_2(X^1\Sigma_g^+)$ ground state. Pathway 2 illustrates the roaming mechanism. The photoexcitation of a CO_2 molecule is indicated by the upward arrows to CO_2 absorption bands manifested by the $C(^3P_2)$ photofragment excitation spectrum in the energy range of 11.655 to 12.212 eV.

the observed $C(^3P_2)$ photofragment excitation spectrum of Fig. 3B shows that the yield of the $C(^3P_2) + O_2(X^3\Sigma_g^-)$ channel decreases as the VUV photodissociation energy decreases toward the thermochemical threshold for the formation of $C(^3P_2) + O_2(X^3\Sigma_g^-)$ at 11.44 eV. These observed dependences of the $C(^3P_2)$ and $O(^1S)$ intensities on the VUV photodissociation energy suggest that the branching ratio of the $C(^3P) + O_2(X^3\Sigma_g^-)$ channel relative to the $O(^1S) + CO(X^1\Sigma^+)$ channel is expected to increase as the VUV photodissociation energy increases. Based on the detection efficiencies for $C(^3P_2)$ and $O(^1S)$ in the present experiment and the measured $C(^3P_{2,1,0})$ fine structure distribution produced from CO_2 photodissociation, we have obtained an estimate of $5 \pm 2\%$ for the intensity of the $C(^3P) + O_2(X^3\Sigma_g^-)$ channel compared with that of the $O(^1S) + CO(X^1\Sigma^+)$ channel formed in CO_2 photodissociation in the current VUV photodissociation energy range near the energetic threshold of the $C(^3P) + O_2(X^3\Sigma_g^-)$ channel (10).

We have measured the $C(^3P_2)$ velocity-map ion images (Fig. 4) at five selected CO_2 predissociative states, as marked by the red downward pointing arrows in Fig. 3B. As shown below, the analysis of these $C(^3P_2)$ ion images provides further support for the direct formation of O_2 molecules in CO_2 photodissociation. The radial distribution of the $C(^3P_2)$ ion image (Fig. 4) provides a measure of the recoil velocity distribution of $C(^3P_2)$ photofragments produced at a specific VUV frequency for CO_2 excitation. The central donut-shaped ring structures of the $C(^3P_2)$ ion images originate from the formation of $C(^3P_2) + O_2(X^3\Sigma_g^-)$ in the photodissociation of CO_2 . As the VUV_1 photon energy increases, the figures show that the radii of the ring structures observed for the $C(^3P_2)$ photofragments increase as expected. Based on the conservation of energy (Eq. 4) and linear momentum, the measurement of the $C(^3P_2)$ photofragments' recoil velocities can be used to determine the E_{trans} distribution of the O_2 counter-fragments and, thus, the TKER of the $C(^3P_2) + O_2(X^3\Sigma_g^-)$ product channel. The TKER spectra (Fig. 4, A to E) exhibit vibrational structures, from which we simulated the vibrational excitations of $O_2(X^3\Sigma_g^-)$ in the range of $v = 0$ to 3, as marked in the figures (10). The best fit to the observed vibrational profiles indicates that $O_2(X^3\Sigma_g^-)$ photofragments are also rotationally excited, with a rotational temperature well above 500 K. The onset energies of the TKER spectra are in excellent agreement with the thermochemical threshold (i.e., $D_0 = 11.44$ eV) for the formation of the $C(^3P_2) + O_2(X^3\Sigma_g^-)$ products from CO_2 photodissociation. The agreement of the $C(^3P_2) + O_2(X^3\Sigma_g^-)$ onset energies and the observation of the $O_2(X^3\Sigma_g^-)$ vibrational distributions are the most unambiguous evidence for the formation of the molecular oxygen channel in the VUV photodissociation of CO_2 . Taking into account the theoretical results, we may rationalize that the production of molecular oxygen at photon energies near the energetic threshold proceeds mostly via the $c-CO_2(^1A_1)$ and $COO(^1\Sigma^+)$ intermediates on the singlet ground-state potential energy surface (pathway 1 in Fig. 1), which is predicted

to have no potential energy barrier by the theoretical calculations (7, 8).

The $C(^3P_2)$ ion image measurements reveal not only the E_{trans} distribution of the photoproduct channels, but also the angular distribution of the photofragments, which is characterized

by the anisotropy β parameter (16). The angular distribution measurements provide information about the photodissociation rates for the formation of specific photofragments, as well as the geometry of the excited molecule at the time of its dissociation. The upper electronic states for the

Fig. 2. Time-of-flight mass spectra from CO_2 photolysis at a VUV_1 energy of 11.832 eV ($95,430.0\text{ cm}^{-1}$).

The top red trace indicates the detection of a C^+ ion from $C(^3P_2)$ with both VUV_1 and VUV_2 turned on. The black and blue traces below represent the background spectra observed by tuning the VUV_2 frequency off resonance and turning off the VUV_1 laser beam, respectively.

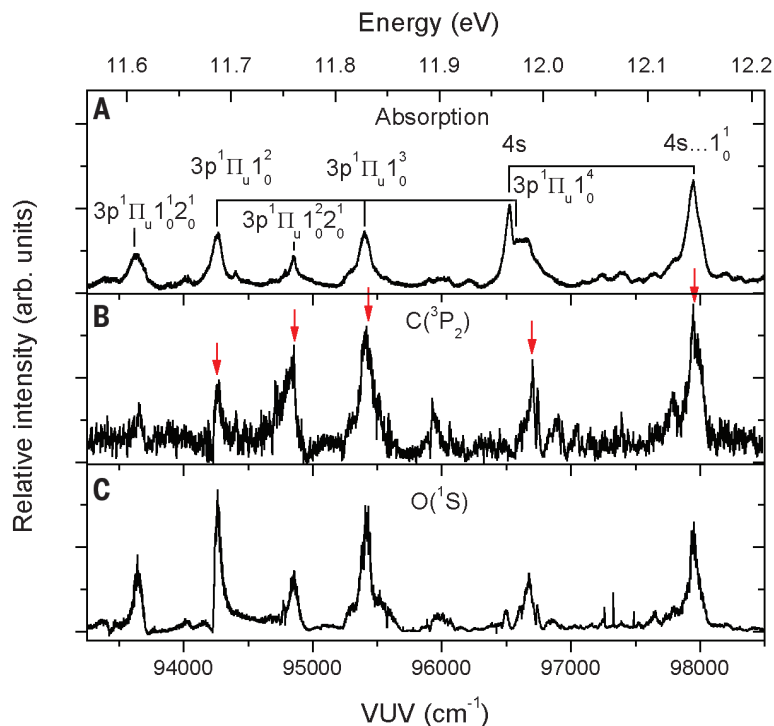
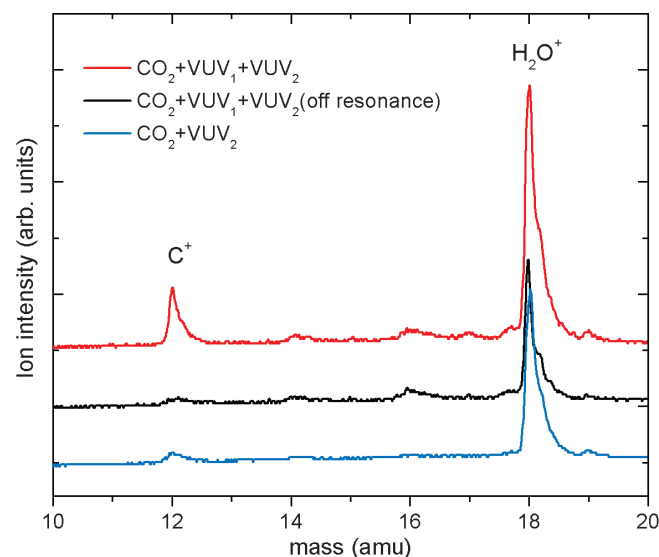


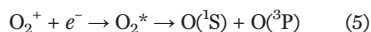
Fig. 3. Photofragment excitation spectra. Comparison of (A) the CO_2 absorption spectrum, (B) the photofragment excitation spectrum for $C(^3P_2)$, and (C) the photofragment excitation spectrum for $O(^1S)$ in the VUV_1 energy region 11.562 to 12.212 eV ($93,250$ to $98,500\text{ cm}^{-1}$) illustrates that the $C(^3P_2)$ and $O(^1S)$ photofragments are observed only when CO_2 is photoexcited to an absorption band. The absorption spectrum was recorded by Archer *et al.* at an optical resolution of 1.15 cm^{-1} (full width at half maximum) (11). The red downward arrows mark the VUV_1 photodissociation energies at which $C(^3P_2)$ ion images were collected. The photofragment excitation spectra shown in (B) and (C) have been normalized by the corresponding VUV_1 laser intensity (14).

images in Fig. 4, A to C, are assigned as $3p^1\Pi_u$ (*12, 13*). Thus, perpendicular distribution of the $C(^3P_2)$ atoms with respect to the CO_2 photolysis laser polarization is expected if CO_2 dissociates in a linear geometry. However, the $C(^3P_2)$ ion images reveal modest parallel distributions with respect to the transition dipole moment in Fig. 4, A to C. This result suggests that the electronically excited CO_2 molecules dissociate in a bent geometry for the formation of $C(^3P_2) + O_2(X^3\Sigma_g^-)$ products due to vibronic interaction. The parallel distribution of the photofragments has been observed in other triatomic molecules in perpendicular transitions due to vibronic interactions (*17–19*). The $3p^1\Pi_u$ Rydberg state of CO_2 is known to involve bending vibrational coupling (*20*), which is caused by the Fermi resonance between the excitation of one quantum of symmetric stretching (ν_1) and two quanta of bending ($2\nu_2$) vibrational modes (*21*). Thus, the inclusion of vibronic interactions would account for the experimental observation of CO_2 dissociation in a bending geometry. The increase of the β value of 0.72 ± 0.07 in Fig. 4C to 0.97 ± 0.09 in Fig. 4D can be attributed to the symmetry change of the upper electronic states from $3p^1\Pi_u$ to $4s$.

The recent experimental and theoretical evidence of a “roaming” dissociation pathway (*22–24*) has prompted us to speculate the involvement of O atom roaming for the formation of $C(^3P_2) +$

$O_2(X^3\Sigma_g^-)$ products in CO_2 photodissociation (pathway 2 in Fig. 1). The roaming mechanism can be initiated via an incomplete OC-O bond cleavage. The resulting molecular CO and atomic O moieties interact via long-range forces on a flat region of the potential energy surface until they encounter a reactive site, leading to the formation of $C + O_2$ products by intramolecular O atom abstraction. Based on the theoretical calculation from Grebenshchikov (*8*), the CO_2 potential energy surfaces are relatively flat for the spin-allowed $CO(a^3\Pi) + O(^3P)$ and $CO(X^3\Sigma^+) + O(^1S)$ dissociation channels. The formation of $C(^3P_2) + O_2(X^3\Sigma_g^-)$ products can result from the O atom roaming with CO on these excited singlet surfaces in CO_2 photodissociation, similar to the roaming dissociation mechanism observed in NO_3 photodissociation (*24*).

Recently, Huestis *et al.* (*25*) suggested that the source of $O(^1S)$ day-glow emission observed from the Mariner 4 and Mars Express missions originates from the dissociative electron-ion recombination reaction (Eq. 5)



even though $O(^1S)$ atoms are known primary photofragments of VUV CO_2 photodissociation in the CO_2 -dominated atmosphere of Mars. More recent measurements by Herschel spacecraft and

Mars Science Laboratory’s Sample Analyses at Mars provide O_2 abundance measurements of 1400 ± 120 parts per million (ppm) (*26*) and 1450 ± 90 ppm (*27*), respectively. The $O(^1S)$ optical emission poses an intriguing question about the source of O_2^+ , which can be produced by VUV photoionization of O_2 and/or charge transfer from CO_2^+ to O_2 in the CO_2 -dominated atmosphere of Mars. Our study has provided unambiguous experimental evidence for the formation of $C + O_2$ photoproducts in CO_2 photodissociation. We suggest that this pathway for generating O_2 be incorporated into future photochemical reaction networks and general circulation models of planetary atmospheres dominated by CO_2 .

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SUPPLEMENTARY MATERIALS

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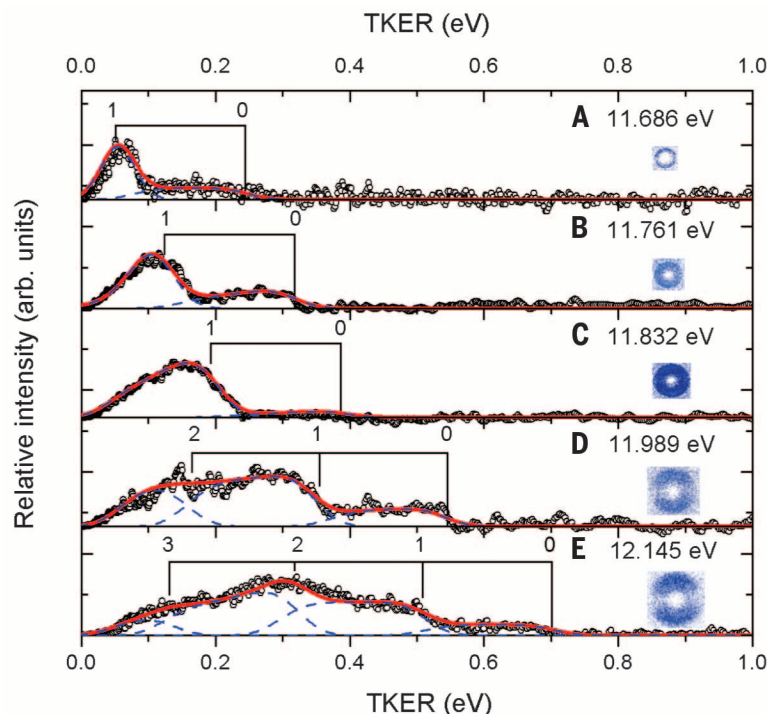


Fig. 4. $C(^3P_2)$ velocity-map ion images and corresponding TKER spectra. VUV₁ photoexcitation of CO_2 was tuned to the absorption bands peaked at the energies indicated above the ion image inserts. TKER spectra are plotted as open circles. Angular anisotropy β parameters derived from these $C(^3P_2)$ ion images are (A) $\beta = 0.45 \pm 0.09$, (B) $\beta = 0.45 \pm 0.08$, (C) $\beta = 0.72 \pm 0.07$, (D) $\beta = 0.97 \pm 0.09$, and (E) $\beta = 1.02 \pm 0.08$. The simulation (red lines) shows that the threshold energies observed are consistent with the known thermochemical threshold of 11.44 eV for the $C(^3P) + O_2(X^3\Sigma_g^-)$ channel, and $O_2(X^3\Sigma_g^-)$ photofragments are formed in $v = 0$ to 3 vibrational levels, as marked on top of the TKER spectra of (A) to (E). The dashed lines show the simulated rotational profiles of individual vibrational bands of $O_2(X^3\Sigma_g^-)$.

MARINE GEOPHYSICS

New global marine gravity model from CryoSat-2 and Jason-1 reveals buried tectonic structure

David T. Sandwell,^{1*} R. Dietmar Müller,² Walter H. F. Smith,³ Emmanuel Garcia,¹ Richard Francis⁴

Gravity models are powerful tools for mapping tectonic structures, especially in the deep ocean basins where the topography remains unmapped by ships or is buried by thick sediment. We combined new radar altimeter measurements from satellites CryoSat-2 and Jason-1 with existing data to construct a global marine gravity model that is two times more accurate than previous models. We found an extinct spreading ridge in the Gulf of Mexico, a major propagating rift in the South Atlantic Ocean, abyssal hill fabric on slow-spreading ridges, and thousands of previously uncharted seamounts. These discoveries allow us to understand regional tectonic processes and highlight the importance of satellite-derived gravity models as one of the primary tools for the investigation of remote ocean basins.

Fracture zones (FZs) spanning the ocean basins reveal the breakup of the continents and the geometry of sea-floor spreading (1). The exact intersection points of the FZs along conjugate continental margins are used for precise reconstruction of the continents (2–4). These FZ intersections are commonly buried by several kilometers of sediments that flow off the continents to fill the voids created by the thermal subsidence of the rifted margins (5). This sediment cover extends hundreds to

thousands of kilometers out onto the oceanic lithosphere, resulting in a relatively flat and featureless sea floor. Reflection seismic profiles can reveal the underlying basement topography of the FZs, but the data coverage is usually insufficient to map out the intersections. In areas of thin sediment cover, the topographic ridges and troughs along the FZs produce large gravity anomalies that are easily traced across the ocean basins (Fig. 1). However, when the topography becomes buried by sediment, the original density contrast of the sea-floor topography is reduced, resulting in more-subdued, and sometimes sign-reversed, gravity signatures (6). Moreover, as the lithosphere ages and cools, the sea floor subsides, causing a blurring of the gravity anomalies; smaller wavelengths of the gravity field become less well-resolved with increasing water depth. Previous global marine gravity models derived from satel-

lite altimetry had sufficient accuracy and coverage to map all FZs in un-sedimented sea floor (7), but the 3 to 5 mGal of gravity noise blurred the small signatures of sediment-covered topography such as seamounts and FZs. Here we report on a new global marine gravity model having ~2-mGal accuracy that is providing a dramatically improved resolution of the 80% of the sea floor that remains uncharted or is buried beneath thick sediment.

Gravity-field accuracy derived from satellite altimetry depends on three factors: altimeter range precision, spatial track density, and diverse track orientation. Two altimeter data sets with high track density have recently become available (CryoSat-2 and Jason-1) to augment the older altimeter data (Geosat and ERS-1), resulting in improvement by a factor 2 to 4 in the global marine gravity field. Their newer radar technology results in a 1.25-times improvement in range precision that maps directly into gravity-field improvement (8). The new altimeters also contribute more than 70 months of data, as compared with the 31 months provided by the older satellites. CryoSat-2 has provided the most dense track coverage, because although it has a nominal 369-day repeat orbit period, the ground tracks are allowed to drift within a 5-km band, so after 4 years in orbit it has provided a nominal track spacing of about 2.5 km. Jason-1 provided 14 months of dense track coverage during its geodetic phase, resulting in a track spacing of 7.5 km.

Most of the improvement in the altimeter-derived gravity field occurs in the 12- to 40-km wavelength band, which is of interest for the investigation of structures as small as 6 km. The current version of the altimeter-derived gravity field has an accuracy of about 2 mGal (8). Unlike terrestrial gravity, where coverage is uneven, these accuracies are available over all marine areas and large inland bodies of water, so this gravity provides an important tool for exploring the deep ocean basins. At scales smaller than 200 km, variations in marine gravity primarily reflect

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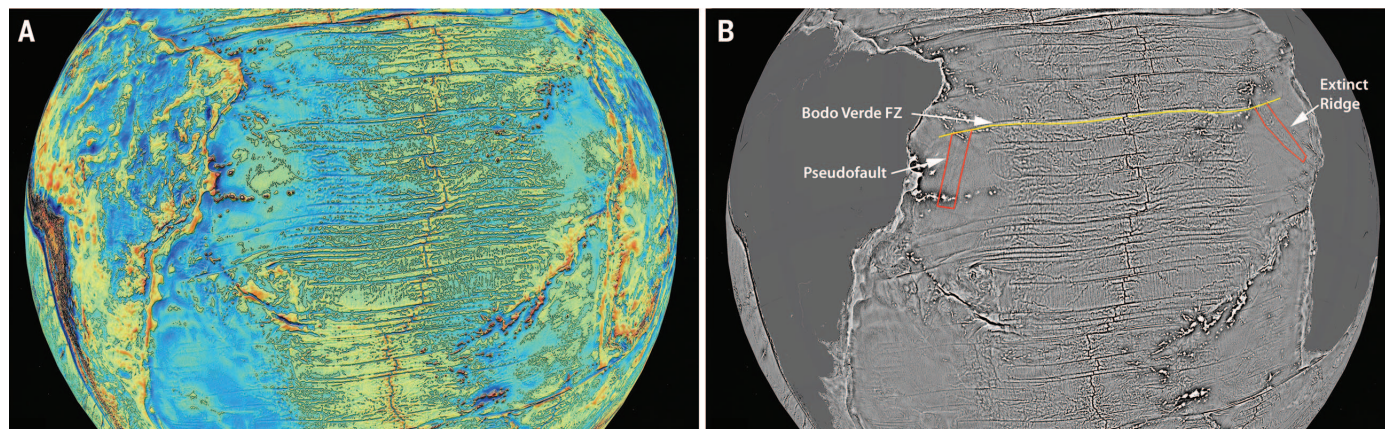


Fig. 1. Ocean gravity maps. (A) New marine gravity anomaly map derived from satellite altimetry reveals tectonic structures of the ocean basins in unprecedented detail, especially in areas covered by thick sediments. Land areas show gravity anomalies from Earth Gravitational Model 2008 (15). **(B)** VGG map derived from satellite altimetry highlights FZs crossing the South Atlantic Ocean basin (yellow line). Areas outlined in red are small-amplitude anomalies in areas

where thick sediment has diminished the gravity signal of the basement topography. The full-resolution gravity anomaly and VGG models can be viewed in Google Earth using the following files: ftp://topex.ucsd.edu/pub/global_grav_1min/global_grav.kmz and ftp://topex.ucsd.edu/pub/global_grav_1min/global_grav_gradient.kmz. The grids are available in the supplementary material, as well as at the following FTP site: ftp://topex.ucsd.edu/pub/global_grav_1min.

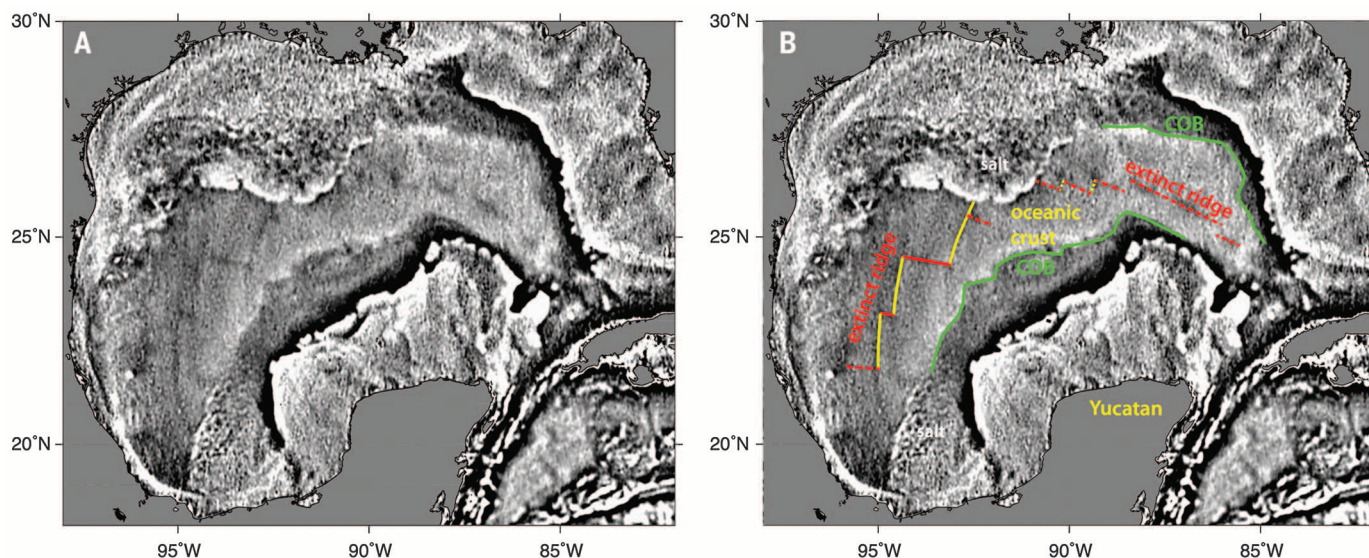


Fig. 2. Gulf of Mexico VGG. (A) Uninterpreted. (B) Our interpretation of tectonic structures, after Pindell and Kennan (9). The VGG reveals subtle signatures of the extinct spreading ridges and FZs as well as a significant change in amplitude across the boundary between continental and oceanic crust (COBs). This is a Mercator projection; grayscale saturates at ± 20 eotvos units.

sea-floor topography generated by plate tectonics such as ridges, FZs, and abyssal hills. Many FZs can now be traced much more closely to the continental margins, and one can also better interpret buried, migrating, unstable FZs, which has the potential to improve the use of FZs as tie points for reconstructions of the boundaries between continental fit reconstructions (Fig. 1). In addition to FZs, there are other tectonic features associated with continental margins, such as the boundaries between continental and oceanic crust [continent-ocean boundaries (COBs)], that can now be mapped in greater detail.

The first example (Fig. 2) is in the Gulf of Mexico, where thick sediments obscure the FZs and extinct ridges. Reconstruction models provide the overall framework of counterclockwise rotation of the Yucatan plate with respect to North America, as well as a generalized position for the COB (9). The new vertical gravity gradient images confirm and refine the positions of these tectonic boundaries. Extinct spreading ridges produce a negative gravity signature, because the relatively high-density sediment cover largely cancels the positive gravity effect of the topographic ridge, leaving the negative gravity signature of the compensating Moho topography (6). In this region, the Moho is more than 15 km beneath the sea surface, so the effects of upward continuation reduce and smooth the anomaly.

The second example is on the African ridge flank, where the new data reveal a major tectonic feature that was not visible in previous satellite gravity data sets because of high-frequency noise. The newly discovered feature is a set of tectonic lineaments roughly between 8°S and 12°S, striking northwest-southeast and obliquely dissected by individual en-echelon faults, stretching from the Bodo Verde Fracture Zone in the north into the middle of the Cretaceous Magnetic Quiet Zone at its southeastern extension (Fig. 1b). This feature

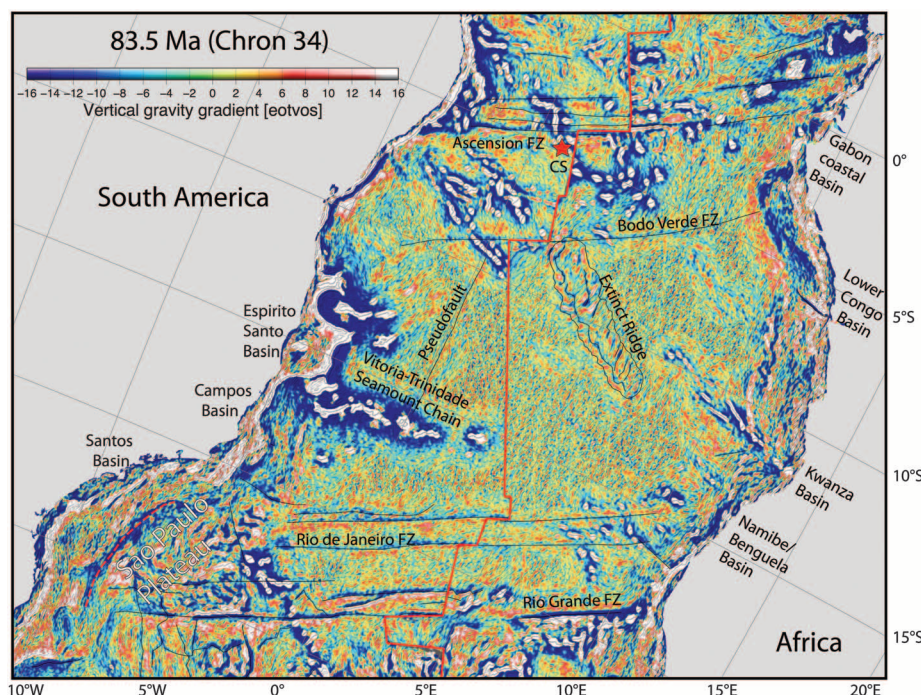


Fig. 3. South Atlantic filtered VGG. Reconstructed at chron 34 (83.5 Ma, orthographic projection) with Africa fixed (16). Major tectonic and volcanic sea-floor features and offshore sedimentary basins are labeled. The mid-ocean ridge is outlined in red, the extinct Abimaël spreading ridge is shown as a dashed red line, and the reconstructed position of the Cardno hot spot (CS) is outlined by a red star. Most of the sea floor shown in this reconstruction was formed during the Cretaceous Normal Superchron. Also note that the extinct Abimaël spreading ridge between the Santos Basin and Sao Paulo Plateau offshore of Brazil is now visible as a negative VGG anomaly (dashed red line), as compared to previous interpretations (17, 18). This region is of great interest for oil and gas exploration, as it is one of the most extensive deepwater oil and gas frontiers globally, with several recent discoveries (19).

is about 800 km long and 100 km wide and does not follow either the azimuth of nearby sea-floor isochrons or FZs. A reconstruction of this feature at magnetic chron 34 [83.5 million years ago (Ma)] (Fig. 3) reveals that it has a mirror-image

counterpart on the South American plate, but this conjugate feature is represented only by a relatively faint gravity lineament (Fig. 3). This feature is visible in the filtered vertical gravity gradient image, marking a boundary between

swaths of differently textured sea-floor fabric to the east and west of the lineament (Fig. 3). The geometry of the two features suggests that they form a pair of an extinct ridge (on the African side) and a pseudofault (on the South American side), created by a northward ridge propagation episode between ~100 and 83 Ma. An absolute hot spot-based plate reconstruction using the rotation parameters from O'Neill *et al.* (10) indicates that the Cardno hot spot (Fig. 3) may have been situated not far north of the northern tip of the ridge propagator, where it abuts the Bodo Verde Fracture Zone; this is where the propagator came to a halt. These observations conform with the inference that ridges have a tendency to propagate toward hot spots/plumes and that propagation events and resulting spreading asymmetries are frequently contained within individual spreading corridors bounded by FZs (11). The existence of major previously unknown ridge propagation events will also be relevant for interpreting marine magnetic anomaly sequences during the Cretaceous Normal Superchron on conjugate ridge flanks (12).

One of the most important uses of this new marine gravity field will be to improve the estimates of sea-floor depth in the 80% of the oceans having no depth soundings. The most accurate method of mapping sea-floor depth uses a multibeam echosounder mounted on a large research vessel. However, even after 40 years of mapping by hundreds of ships, one finds that more than 50% of the ocean floor is more than 10 km away from a depth measurement. Between the soundings, the sea-floor depth is estimated from marine gravity measurements from satellite altimetry (13). This method works best on sea floor where sediments are thin, resulting in a high correlation between sea-floor topography and gravity anomalies in the 12-km-to-160-km wavelength band. The shorter wavelengths are attenuated because of Newton's inverse square law, whereas the longer wavelengths are partially cancelled by the gravity anomalies caused by the isostatic topography on the Moho (13). The abyssal hill fabric created during the sea-floor spreading process has characteristic wavelengths of 2 to 12 km, so it is now becoming visible in the vertical gravity gradient (VGG) models, especially on the flanks of the slower-spreading ridges (14). Additionally, seamounts between 1 and 2 km tall, which were not apparent in the older gravity models, are becoming visible in the new data. As CryoSat-2 continues to map the ocean surface topography, the noise in the global marine gravity field will decrease. Additional analysis of the existing data, combined with this steady decrease in noise, will enable dramatic improvements in our understanding of deep ocean tectonic processes.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/346/6205/65/suppl/DC1
Supplementary Text
Figs. S1 and S2
References (20–33)

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NANOPHOTONICS

Chiral nanophotonic waveguide interface based on spin-orbit interaction of light

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Controlling the flow of light with nanophotonic waveguides has the potential of transforming integrated information processing. Because of the strong transverse confinement of the guided photons, their internal spin and their orbital angular momentum get coupled. Using this spin-orbit interaction of light, we break the mirror symmetry of the scattering of light with a gold nanoparticle on the surface of a nanophotonic waveguide and realize a chiral waveguide coupler in which the handedness of the incident light determines the propagation direction in the waveguide. We control the directionality of the scattering process and can direct up to 94% of the incoupled light into a given direction. Our approach allows for the control and manipulation of light in optical waveguides and new designs of optical sensors.

The development of integrated electronic circuits laid the foundations for the information age, which fundamentally changed modern society. During the past decades, a transition from electronic to photonic information transfer took place, and nowadays, nanophotonic circuits and waveguides promise to partially replace their electronic counterparts and to enable radically new functionalities (1–3). The strong confinement of light provided by such waveguides leads to large intensity gradients on the wavelength scale. In this strongly nonparaxial regime, spin and orbital angular momentum of

light are no longer independent physical quantities but are coupled (4, 5). In particular, the spin depends on the position in the transverse plane and on the propagation direction of light in the waveguide—an effect referred to as spin-orbit interaction of light (SOI). This effect holds great promises for the investigation of a large range of physical phenomena such as the spin-Hall effect (6, 7) and extraordinary momentum states (8) and has been observed for freely propagating light fields (9, 10) in the case of total internal reflection (11, 12), in plasmonic systems (13–15), and for radio frequency waves in metamaterials (16). Recently, it has been demonstrated in a cavity-quantum electrodynamics setup in which SOI fundamentally modifies the coupling between a single atom and the resonator field (17).

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For vacuum-clad dielectric waveguides, evanescent fields arise in the vicinity of the surface and allow one to locally interface the guided fields with micro- and nanoscopic emitters (18). Because of SOI, these evanescent fields exhibit a locally varying ellipticity that stems from a longitudinal polarization component that points in the direction of propagation of the light and that oscillates in quadrature with respect to the transversal components (19). Surprisingly and in contrast to paraxial light fields, the corresponding photon spin is in general not parallel or antiparallel to the propagation direction of the guided light. In special cases, it can even be perpendicular to the propagation direction and antiparallel to the orbital angular momentum (8, 20).

We have experimentally demonstrated that SOI in a dielectric nanophotonic waveguide drastically changes the scattering characteristics of a nanoscale particle located in the waveguide's evanescent field. In free space, pointlike scatterers exhibit a dipolar emission pattern (21) in which for the emission of both linearly and circularly polarized light, the intensity distribution of the scattered light is cylindrically symmetric. In particular, this implies that dipolar scattering into any spatial direction perpendicular to the symmetry axis is always accompanied by an equal amount of scattered light into the opposite direction. We demonstrate that SOI breaks this symmetry and show that when light is scattered by the particle into the waveguide modes, the amount of light that is coupled into a given

direction of the waveguide can substantially exceed the power that propagates in the opposite direction.

We used an air-clad silica nanofiber as an optical waveguide and positioned a single spherical gold nanoparticle on its surface. We illuminated the particle with a focused paraxial laser beam from the side (Fig. 1A) and characterized the scattering properties of the particle into the optical waveguide. The emission rate of the particle into a given nanofiber eigenmode is proportional to $|\mathbf{d}^* \cdot \boldsymbol{\epsilon}|^2$, with the induced electric dipole moment $\mathbf{d}$ of the particle and the profile function $\boldsymbol{\epsilon}$ of the electric part of the fiber mode (22). For spherical scatterers, the dipole moment is $\mathbf{d} = \alpha \cdot \mathbf{E}_{\text{exc}}$, where α is the complex polarizability and $\mathbf{E}_{\text{exc}}$ is the positive-frequency envelope of the excitation field, which is related to the real value of the electric field by $\mathbf{E}_{\text{exc}} = 1/2[\mathbf{E}_{\text{exc}} \exp(-i\omega t) + c.c.]$. Here, $\omega/2\pi$ is the frequency of the light, and *c.c.* is the complex conjugate. The total power of the light scattered into a given fiber mode is given by

$$I_{\text{scat}} \propto |\mathbf{d}^* \cdot \boldsymbol{\epsilon}(r, \phi)|^2 = |\alpha^* \mathbf{E}_{\text{exc}} \cdot \boldsymbol{\epsilon}(r, \phi)|^2 \quad (1)$$

where (r, ϕ) denotes the position of the scatterer in the nanofiber transverse plane. As a consequence, the emission rate is directly proportional to the overlap between the field of the excitation light and the fiber mode at the particle's position.

For a single-mode nanofiber, all guided light fields can be decomposed into the quasi-linearly

polarized fiber eigenmodes $(19) HE_{11,x}^{\pm}$ and $HE_{11,y}^{\pm}$, where the z axis coincides with the nanofiber axis and the $\pm$ sign indicates the propagation direction ($\pm z$) of the light in the fiber. We choose $HE_{11,x}^{\pm}$ and $HE_{11,y}^{\pm}$ so that their main polarization component points along the x ($\phi = 0^\circ$) and y ($\phi = 90^\circ$) directions, respectively. The normalized total power of the light scattered into the $HE_{11,x}^{\pm}$ and $HE_{11,y}^{\pm}$ modes is shown in Fig. 2, according to Eq. 1, as a function of the position of the scatterer in the fiber transverse plane. The calculations were performed for circularly $\sigma^{\pm} = (i\mathbf{e}_z \pm \mathbf{e}_y)/\sqrt{2}$ and linearly $\pi = \mathbf{e}_x$ polarized excitation light. Here, the x axis is the quantization axis, and $\mathbf{e}_{x,y,z}$ are the unit vectors along the corresponding axes. The predicted asymmetry of the scattering originates from the fact that the local polarization depends both on the position in the fiber transverse plane and on the propagation direction of the mode—a consequence of SOI of the nanofiber-guided light. For a particle located at the top ($\phi = 90^\circ$) of the nanofiber, the polarization overlap between the $HE_{11,y}^+$ mode and σ^- is maximal and reaches 93%, and the overlap between the $HE_{11,y}^-$ mode and σ^- is minimal and reaches 7%. This means that the field is nearly perfectly circularly polarized. Because the emission probability of the particle into the fiber is directly proportional to this overlap, a strong asymmetry of the scattering into the left ($+z$) and right ($-z$) direction of the fiber results, which can be tuned by the polarization of the incident light field and the position of the nanoparticle. In particular, the asymmetry reverses when switching

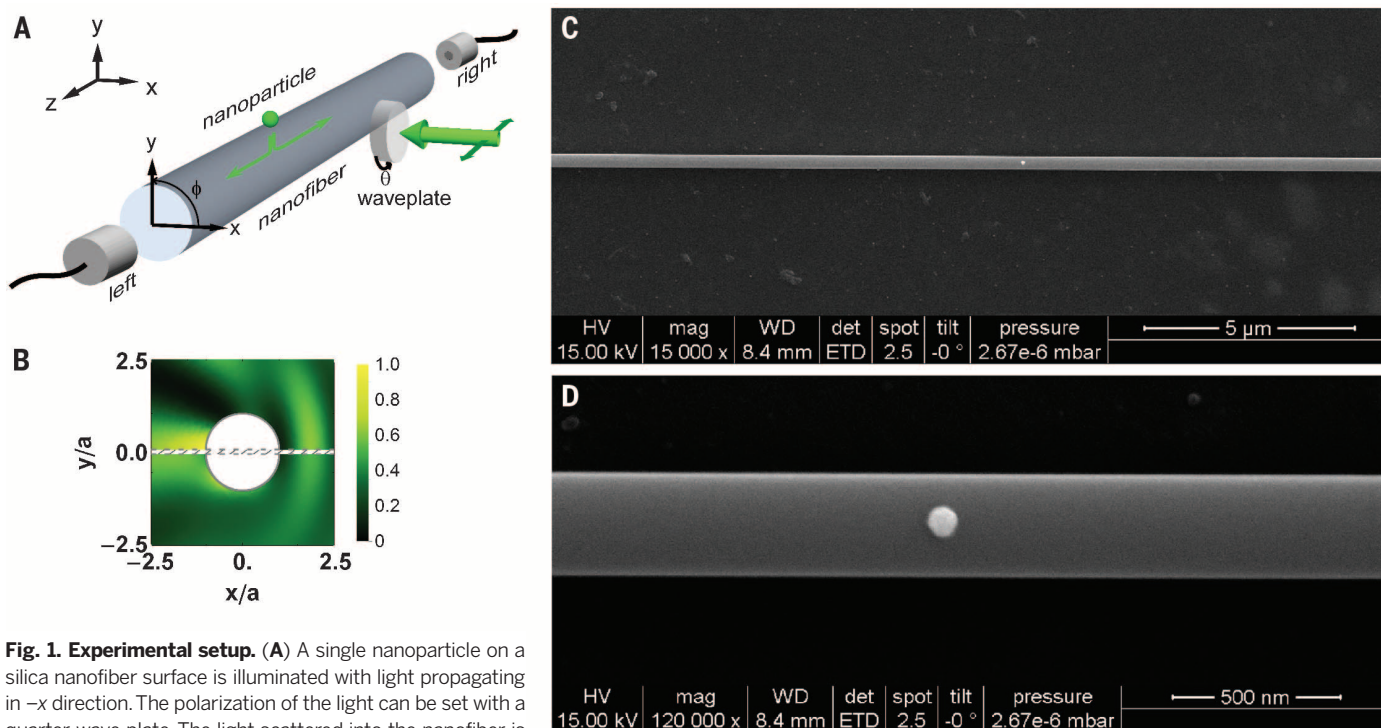


Fig. 1. Experimental setup. (A) A single nanoparticle on a silica nanofiber surface is illuminated with light propagating in $-x$ direction. The polarization of the light can be set with a quarter-wave plate. The light scattered into the nanofiber is detected by using SPCMs at the left and right fiber output ports. (B) Modification of the intensity distributions for an incident field polarized along y (bottom half) and z axis (top half) owing to the presence of the nanofiber. (C and D) Scanning electron microscope images of the nanofiber and the nanoparticle used in our experiments. From the images, we determine diameters of $2a = (315 \pm 3)$ nm for the fiber and $2r = (90 \pm 3)$ nm for the nanoparticle.

Fig. 2. Scattering in the presence of SOI (theoretical predictions). (A) When the nanofiber-guided light is quasi-linearly polarized along the y axis, longitudinal polarization components occur. For light traveling in $+z$ direction, this leads to nearly circular σ^- polarization on the top of the fiber and σ^+ polarization on the bottom of the fiber (circular green arrows). For light propagating in $-z$ direction, $\sigma^- = (ie_z - e_y)/\sqrt{2}$ and $\sigma^+ = (ie_z + e_y)/\sqrt{2}$ are interchanged. At these positions, the spin angular momentum of the light (yellow arrows) is oriented perpendicular to the propagation direction and antiparallel to the orbital angular momentum (red arrows), defined with respect to the z axis (8). (B) Intensity, $|e_{HE,y}^\pm|^2$, of the $HE_{11,y}^\pm$ modes, normalized to its peak value on the fiber axis. (C) Position-dependent power, scattered into the $HE_{11,y}^+$ mode for σ^- -polarized excitation light and into the $HE_{11,y}^-$ mode for σ^+ polarized excitation light. (D) Power scattered into the $HE_{11,y}^+$ mode for σ^+ polarization and $HE_{11,y}^-$ mode for σ^- polarization. (E) Power scattered into the $HE_{11,y}^\pm$ modes for $\pi = e_x$ polarization. (F to I) Same as (B) to (E) but for the fiber modes $HE_{11,x}^\pm$. The calculations assume our experimental parameters, and the scattered powers are normalized to the peak value on the fiber axis in (I). On top of the fiber ($x = 0, y = a$), we find a normalized power of 0.88, 0.06, and 0 in panels (C) to (E), respectively.

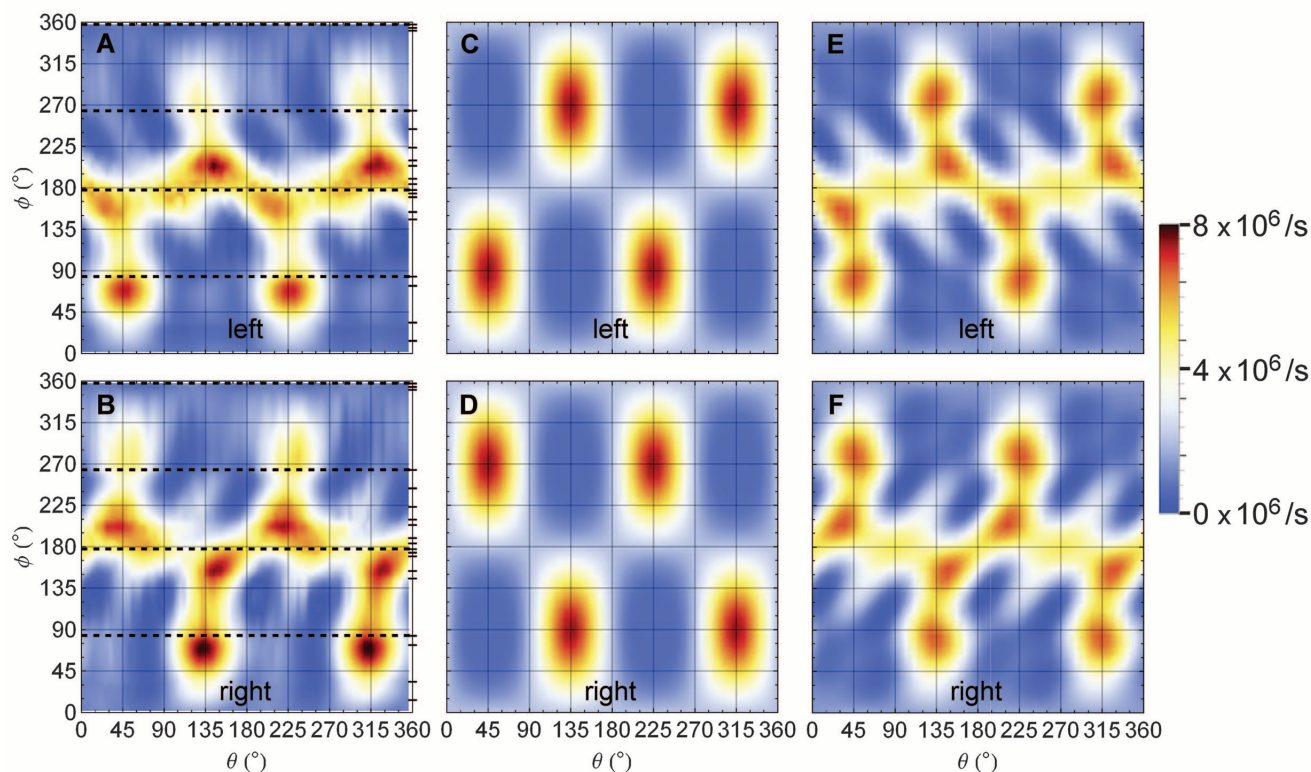
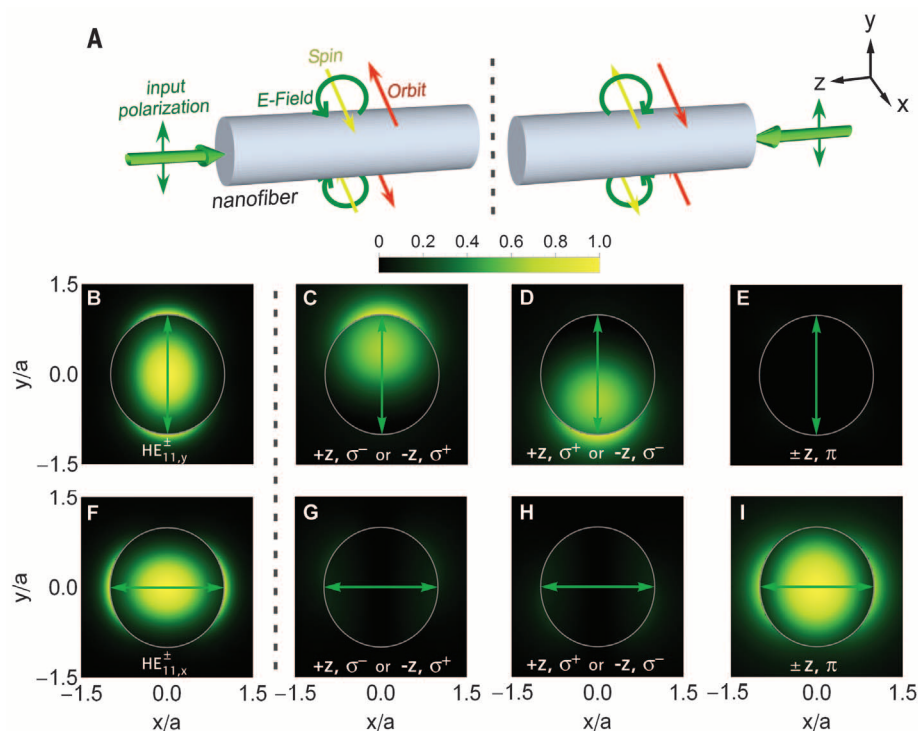


Fig. 3. Chiral waveguide coupling: experiment and theory. (A and B) Measured photon flux (raw data, only corrected for the nonlinear response of the SPCMs) of the light scattered into the (A) left and (B) right direction as a function of the azimuthal position of the nanoparticle ϕ and the polarization of the excitation light field set by the angle θ of the quarter-wave plate. The ticks on the right mark the azimuthal positions for which data have been acquired with a stepsize of θ of 5° .

The data are interpolated in between the measured points. The dashed lines indicate the data sets plotted in Fig. 4. (C to F) Theoretical prediction for the photon fluxes when [(C) and (D)] neglecting and [(E) and (F)] including the effect of the nanofiber on the incident light field. The model uses the angular offset of the nanoparticle and the overall amplitude of the photon flux as free parameters, which are obtained from a fit of (E) and (F) to the data in (A) and (B) (22).

the polarization of the excitation light from σ^- to σ^+ or when changing the position of the particle from (x, y) to $(x, -y)$.

We investigated this directional scattering using a tapered optical fiber (TOF) with a nanofiber waist (23) [diameter, $2a = (315 \pm 3)$ nm], which enables almost lossless coupling of light from a standard optical fiber into and out of the nanofiber section. A single spherical gold nanoparticle [diameter, $2r = (90 \pm 3)$ nm] positioned on the nanofiber surface (22) acts as a polarization-maintaining scatterer (24, 25). The particle is illuminated with a laser beam propagating in the $-x$ direction (Fig. 1A) with a wavelength of 532 nm, which is close to the measured resonance of the nanoparticle at 530 nm (full width at half maximum, 50 nm). The nanofiber can be rotated around the z axis, which because of its cylindrical symmetry amounts to changing the azimuthal position ϕ of the nanoparticle around the fiber (Fig. 1A). The polarization of the incident light field is set by means of a quarter-wave plate. The angle θ between its optical axis and

the y axis can be adjusted at will. Before passing through the waveplate, the polarization of the light is aligned along z . Thus, we can set the polarization to linear along z ($\theta = 0^\circ, 90^\circ$) and circular, σ^- ($\theta = 45^\circ$) or σ^+ ($\theta = 135^\circ$). For intermediate angles, the polarization is elliptical, with the major axis along z . The excitation laser beam has a waist radius of around $w = 150$ μm at the position of the nanoparticle, assuring a homogeneous spatial intensity distribution with negligible longitudinal polarization components. A single-photon counting module (SPCM) at each output port of the TOF detects the light scattered into the nanofiber. After completion of all measurements, we analyzed the fiber surface with a scanning electron microscope (Fig. 1, C and D) to check that only a single nanoparticle was present and to measure the diameters of fiber and nanoparticle.

The measured photon fluxes at both fiber outputs are shown in Fig. 3, A and B, as a function of the azimuthal position of the nanoparticle and the polarization of the excitation light field, and

the theoretical predictions are shown in Fig. 3, C and D, calculated according to Eq. 1 under the assumption that the polarization and intensity distribution of the incident light field are not modified by the presence of the optical fiber (22). We find qualitative agreement between measurement and theoretical prediction. In particular, we observed the expected maximum of the left-right asymmetry for the case of circular input polarization with the particle located at the top or the bottom of the fiber. However, scattering and refraction of the excitation light field by the nanofiber led to an appreciable modification of the polarization and intensity of the field close to the nanofiber surface (Fig. 1B) (26). Including these effects, we obtained the theoretical predictions shown in Fig. 3, E and F, where we used two fit parameters: the angular offset $\phi_0 = 6.3^\circ \pm 0.1^\circ$ of the nanoparticle from the expected deposition position of $\phi = 90^\circ$ and the amplitude $\kappa_f = (21.0 \pm 0.1) \times 10^6 \text{ s}^{-1}$ of the photon flux detected with the SPCMs (22). This model agrees well with the measured data. The main differences to the simple model are an increase of the scattering rate around $\phi = 180^\circ$ owing to the focusing of the incident light field by the fiber and the emergence of a shadow region around $\phi = 120^\circ$ and $\phi = 240^\circ$, with a concomitant decrease in the scattering rate.

For closer comparison, Fig. 4, A to D, shows the polarization dependence of the measured photon flux in the fiber for selected azimuthal positions of the nanoparticle together with the theoretical prediction. For the cases of the nanoparticle positioned near the top and the bottom of the nanofiber, we also plot the directionality

$$\mathcal{D} = \frac{c_+ - c_-}{c_+ + c_-} \quad (2)$$

of the scattering process together with the theoretical prediction (Fig. 4, E and F), where c_+ is the photon flux detected by the left detector and c_- is the photon flux detected by the right detector. We observed a maximum directionality of $\mathcal{D} = 0.88$ for a particle near the top of the fiber, which corresponds to a ratio of 16:1 between the photon flux scattered to the left and right, and $\mathcal{D} = 0.95$ for a particle near the bottom of the fiber, which corresponds to a ratio of 40:1 between the photon flux scattered to the right and left. When the particle is located near the side of the fiber, the overlap of the fiber eigenmodes with any polarization of the excitation light is independent of the propagation direction, and zero directionality is expected. In the experiment, we indeed observed only a small variation with the incident polarization (Fig. 4, A and B). The residual modulation most probably is due to the small angular deviation of the nanoparticle position from the ideal point.

The underlying physical mechanism that enables the directional scattering is spin-orbit interaction of light, which universally occurs in light fields that are strongly confined in the transversal direction. Our method is thus highly versatile, and we expect it to find application in various scenarios of nanophotonic systems. There

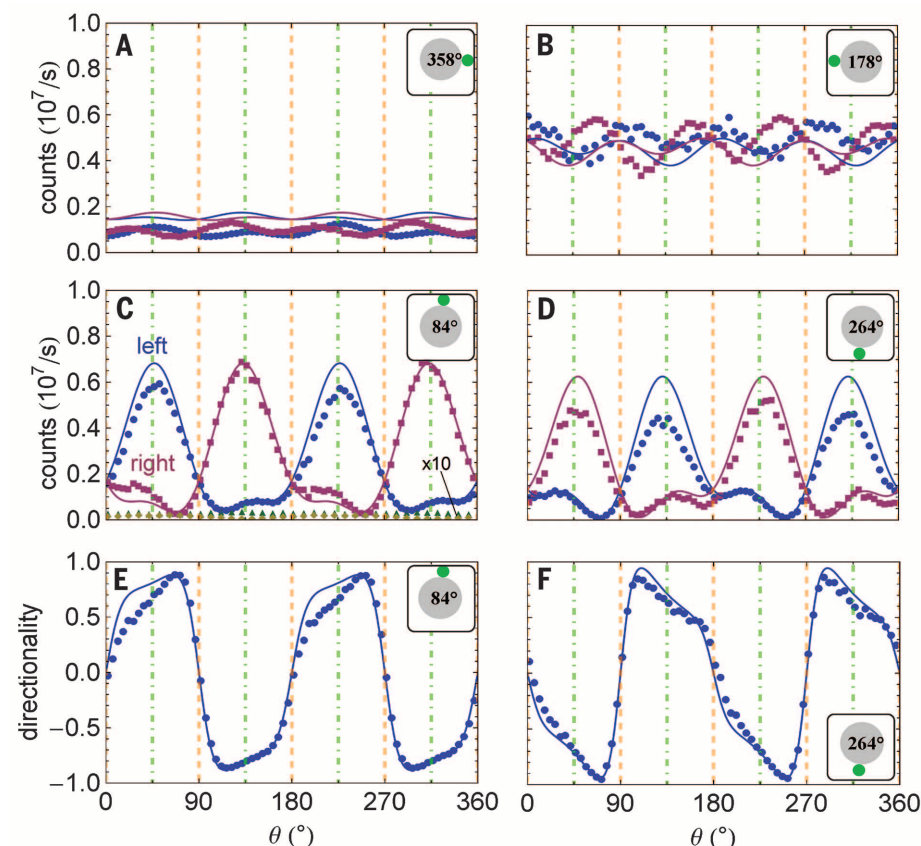


Fig. 4. Directionality of the scattering process. (A to D) Measured photon fluxes at the left (blue circles) and right (purple squares) fiber output ports as a function of the angle θ of the quarter-wave plate. Here, $\theta = 0^\circ, 90^\circ, \dots$ corresponds to linear polarization along z (dashed orange lines), $\theta = 45^\circ, 225^\circ$ corresponds to σ^- polarization of the incident light field, and $\theta = 135^\circ, 315^\circ$ corresponds to σ^+ polarization of the incident light field (dash-dotted green lines). (A) to (D) correspond to the azimuthal positions ($\phi = 358^\circ, 178^\circ, 84^\circ$, and 264°) of the nanoparticle (green dot) around the fiber (gray disk), as indicated in the insets. The solid lines are the predictions of our theoretical model. The statistical error bars are too small to be visible in the plot. The measured photon fluxes to the left (yellow diamonds) and right (green triangles) also are shown in (C) for the nanofiber without the nanoparticle, scaled up by a factor of 10. (E and F) Directionality of the scattering process into the fiber for the data in (C) and (D).

is no fundamental limit to the directionality: By setting the polarization of the excitation field orthogonal to the polarization of the fiber eigenmodes that copropagate into the left/right direction, unity directionality can always be realized (22). Moreover, at the inside of the waveguide, the quasi-linearly polarized guided modes of our silica nanofiber exhibit a perfectly circular polarization at two specific positions in the fiber transverse plane. Thus, a particle at such a position that is excited with circularly polarized light will couple light exclusively into one direction of the waveguide. Apart from their usefulness for optical signal processing and routing of light, our findings have important consequences for the interaction between atoms and light in evanescent fields (27, 28) or strongly focused laser beams. Moreover, they may enable novel nanophotonic sensors that allow one to detect and identify, for example, scatterers with an intrinsic polarization asymmetry (22, 29). In the course of preparing this manuscript, we became aware of two related theoretical works (30, 31) discussing effects based on directional emission in photonic crystal waveguides.

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BIOFUELS

Engineering alcohol tolerance in yeast

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Ethanol toxicity in the yeast *Saccharomyces cerevisiae* limits titer and productivity in the industrial production of transportation bioethanol. We show that strengthening the opposing potassium and proton electrochemical membrane gradients is a mechanism that enhances general resistance to multiple alcohols. The elevation of extracellular potassium and pH physically bolsters these gradients, increasing tolerance to higher alcohols and ethanol fermentation in commercial and laboratory strains (including a xylose-fermenting strain) under industrial-like conditions. Production per cell remains largely unchanged, with improvements deriving from heightened population viability. Likewise, up-regulation of the potassium and proton pumps in the laboratory strain enhances performance to levels exceeding those of industrial strains. Although genetically complex, alcohol tolerance can thus be dominated by a single cellular process, one controlled by a major physicochemical component but amenable to biological augmentation.

The increased use of renewable transportation fuels such as bioethanol is an accepted strategy to combat global climate change (1). However, the toxicity of ethanol and other alcohols to the yeast *Saccharomyces cerevisiae* is a primary factor limiting titer and productivity in industrial production (2, 3). Ethanol tolerance is a complex phenotype: Studies have shown that no single genetic modification is capable of eliciting greater resistance at high ethanol levels (4–7).

Because toxicity may arise from chemical perturbation of the plasma membrane, we surmised that the ionic composition of the culture medium could play a role in exacerbating or ameliorating this destabilization (8–10). Therefore, we added various salts to high-cell-density and high-glucose cultures mimicking industrial fermentation to ascertain their effects on ethanol production.

Monopotassium phosphate (K-P_i) added to standard yeast synthetic complete (YSC) medium induced the greatest improvement (fig. S1), an effect that we dissected into components deriving from elevated potassium (K⁺) and pH. Specifically, when the pH of cultures containing elevated potassium chloride (KCl) was manually adjusted with potassium hydroxide (KOH) throughout the course of fermentation to match that of cultures

containing elevated K-P_i, ethanol titers were statistically indistinguishable ($P = 0.09$ from a two-sample t test; $P \leq 7.6 \times 10^{-3}$ for other pairs) from one another (figs. S2 and S3). We also determined that KCl elicited a statistically higher improvement than sodium chloride (NaCl), and that supplementation with NaCl and sodium hydroxide, or with monosodium phosphate, demonstrated a distinguishable boost over NaCl alone ($P \leq 2 \times 10^{-4}$ from pairwise t tests). Thus, the greatest improvements in ethanol production derived specifically from the increase in K⁺ concentration and the reduction in acidity of the fermentation medium.

Over the course of a 3-day culture, supplementation with KCl and KOH enhanced ethanol titer and volumetric productivity (grams of ethanol per volume per hour), two key benchmarks of fermentative performance (Fig. 1A). Additionally, compared with equimolar KCl or matched pH alone, the combination of K⁺ supplementation and acidity reduction enabled the complete utilization of glucose and decreases in the synthesis of acetic acid and glycerol, two undesired byproducts of fermentation (fig. S4, A to D). Ethanol titers of 115 to 120 g/liter have been reported previously from S288C (11), the inbred laboratory strain we used that is known for its low ethanol resistance (5, 12, 13). However, these studies were typically conducted using chemically undefined ("rich") media. The 128 ± 0.7 (SD) g/liter concentrations we observed were achieved using a purely synthetic formulation, allowing us to identify and precisely control the environmental components that affect ethanol tolerance.

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The boost in ethanol production from KCl and KOH supplementation did not arise simply from an increase in cell number but from an increase in cell tolerance. Specifically, the $80 \pm 1.3\%$ jump in titer (Fig. 1A) was accompanied only by an $11 \pm 4.6\%$ average higher cell density (Fig. 1B); therefore, cell growth alone could not explain the rise in output. This discrepancy, however, was resolved when we directly assessed the fractions of cells remaining alive throughout fermentation (fig. S5A) and discovered that the addition of KCl and KOH enhanced overall population viability (Fig. 1B and fig. S5B). This enhancement, furthermore, occurred despite the increase in toxicity imposed by higher accumulations of ethanol.

When we calculated specific productivities (rates of ethanol increase normalized by the live, rather than total, cell population), the values from KCl and KOH supplementation differed from the control by an average of $11 \pm 7.5\%$ (Fig. 1A). The fact that these differences account for a minor portion of the increase in titer suggests that elevated K^+ and pH act primarily not by affecting per-cell output but by boosting tolerance and the overall viable cell population. Additionally, these effects were fully observed in fermentations con-

ducted in anaerobic bioreactors, demonstrating that these tolerance improvements do not depend on oxygen availability and can scale to higher-volume environments (fig. S6).

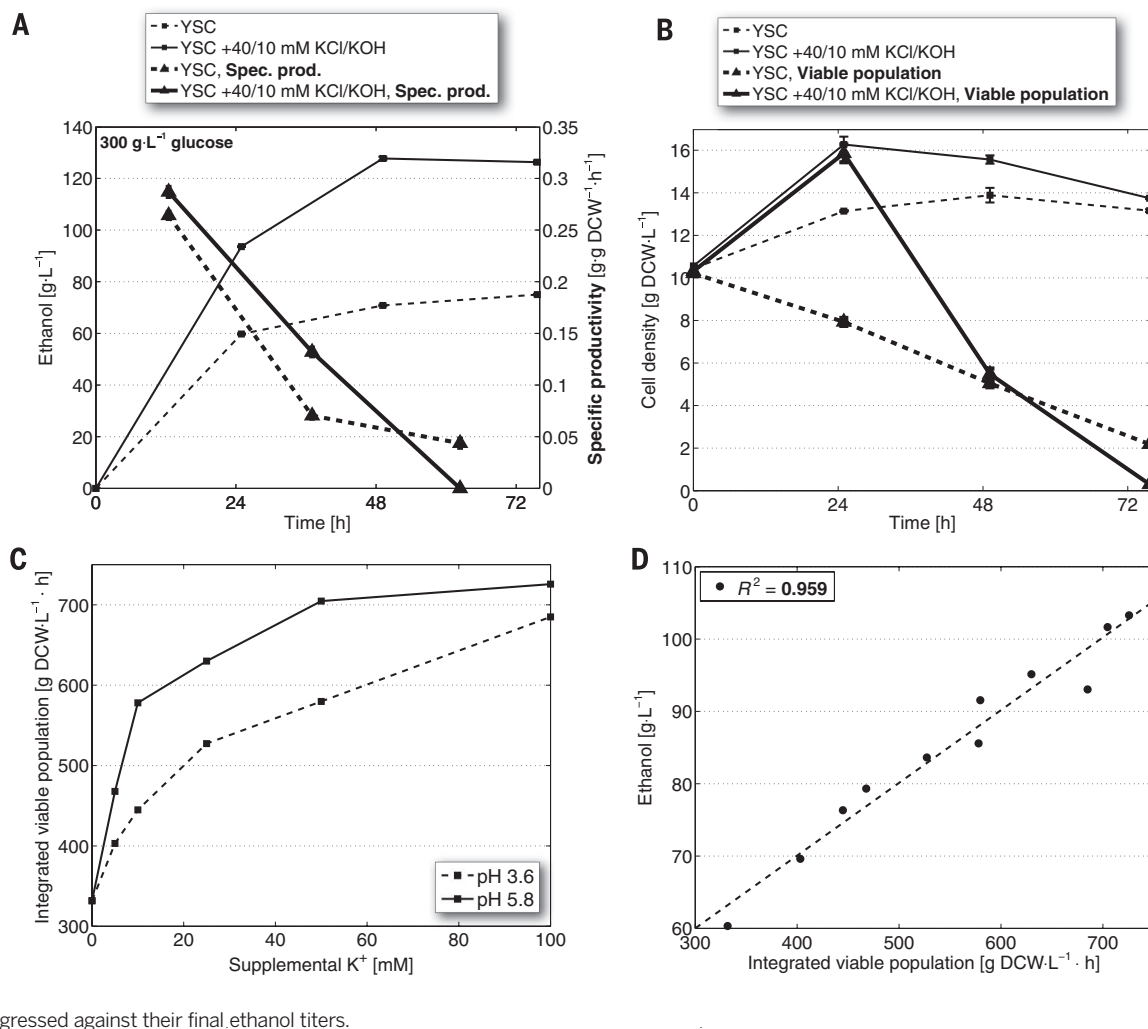
Because it is the viable cell population that is actively fermenting, final titers are governed by both the number of live cells and the length of time over which cells maintain viability against rising ethanol toxicity. The time integral of the viable cell population captures these two aspects and quantifies the overall impact of tolerance on ethanol production (fig. S5, B and C). Indeed, varying the concentration of supplemental KCl resulted in a dose-dependent increase of these time integrals; moreover, when these same additions were done at a higher pH, integrated viable population values were shifted upward correspondingly (Fig. 1C and fig. S7A). A strong linear relationship exists between the time integrals of viable cell density and final titers [goodness-of-fit (R^2) = 0.96; $P = 1.5 \times 10^{-7}$], demonstrating that the ability to endure toxicity is a principal determinant of ethanol output and the primary trait strengthened by KCl and KOH supplementation (Fig. 1D and fig. S7B).

The enhancements conferred by elevated K^+ and reduced acidity transcend genetic back-

ground and were elicited universally among a random sampling of industrial yeast strains. Those used in the production of biofuel ethanol in Brazil (PE-2) and the United States (Lasaffre Ethanol Red), and of sake wine in Japan (Kyokai No. 7), are typically the result of genetic selection efforts designed to isolate superior ethanol phenotypes (13–15). Consequently, all demonstrated distinctly higher ethanol output than S288C [$10 \pm 1\%$ to $30 \pm 1.2\%$] when grown in unmodified medium (Fig. 2A). However, when subjected to KCl and KOH supplementation, all strains responded with enhancements in tolerance that enabled the complete consumption of glucose (fig. S8) and titers of 116 ± 0.9 to 127 ± 1.6 g/liter. Under these conditions, S288C performed indistinguishably from the two industrial bioethanol strains ($P \geq 0.08$ from pairwise t tests). Thus, a strain traditionally deemed ethanol-sensitive is inherently capable—without genetic modification—of superior tolerance, indicating that K^+ supplementation and acidity reduction drive a process that can supersede advantages conferred by genetic adaptation.

These adjustments to the medium, furthermore, enhance fermentation from xylose, an important hemicellulosic sugar that cannot be consumed by

Fig. 1. Elevated extracellular potassium and pH enhance ethanol tolerance and production under high-glucose and high-cell-density conditions. (A) Ethanol titers (squares) and per-cell rates of production (triangles) from fermentations in unmodified YSC medium (dashed lines) or YSC supplemented with 40 mM KCl and 10 mM KOH (solid lines). Specific productivities are calculated from the mean viable population [thick lines from (B)] during each 24-hour period. DCW, dry cell weight. (B) Cell densities (DCW; thin squares) and the underlying viable populations (thick triangles) from the fermentations in (A). Data are mean $\pm$ SD from three biological replicates. (C) Net fermentation viability, expressed as time integrals of the viable cell population, as a function of potassium added to YSC in the form of KCl (pH 3.6), or 5 mM KOH + KCl (pH 5.8). (D) Time integral values from (C) regressed against their final ethanol titers.



standard strains of *S. cerevisiae*. In an engineered strain (16), 22 ± 0.9 g/liter ethanol was produced from unmodified medium containing 100 g/liter of xylose (Fig. 2A). When fermented with the addition of KCl and KOH, we observed a $54 \pm 5.7\%$ increase in titer, commensurate with the complete assimilation of xylose (fig. S8). Thus, K^+ supplementation and acidity reduction enhance tolerance in a manner impartial to the type of substrate.

The improvements conferred by elevated K^+ and pH generalize beyond synthetic media to chemically undefined broths, provided that such formulations do not already saturate for these effects. For example, in yeast extract peptone (YP) medium [$\sim$ pH 6 and unknown concentrations of individual nutrients (17)], cells ferment all sugar, so no margin is available for improvement (fig. S9B). However, the impact of specific supplements can be assessed if the YP components are made limiting. Indeed, when YP was decreased to 30 or 3% while maintaining the same glucose concentration, supplementation with K^+ improved ethanol output, whereas additives shown to be fermentation-neutral (fig. S1) did not (fig. S9A). Using YP diluted to 20%, titers of 104 ± 0.8 g/liter were produced, whereas the addition of K^+ enhanced output $17 \pm 2.5\%$ (Fig. 2B). When pH was reduced from 6 to 3.7, production was concomitantly reduced $28 \pm 0.8\%$. However, the subsequent addition of K^+ compensated for this decrease, restoring titers to 109 ± 1.8 g/liter.

Thus, in media with undefined composition, extracellular K^+ and pH are also sufficient to quantitatively modulate ethanol performance.

To isolate the effects of KCl and KOH supplementation on tolerance from other fermentation variables (such as decreasing turgor pressure from glucose consumption), we subjected yeast to non-physiological step increases in ethanol concentration and quantified the population fractions surviving after 80 min, a period much shorter than the length of fermentation but adequate for cell viability to be affected. In medium containing a subsistence amount of glucose that minimizes newly produced ethanol, elevated K^+ and pH enhanced viability in shocks up to 27% (vol/vol) when compared to cells stressed in unmodified conditions (Fig. 2C). Analogous experiments performed using high glucose (mimicking the osmotic conditions of high-gravity fermentation) and heightened $K-P_i$ yielded a similar result, albeit at a lower range of ethanol concentrations (fig. S10A). These results indicate that the impact of elevated K^+ and reduced acidity is relatively immediate, does not require adaptation to ethanol accumulation over the course of days, and is capable of overcoming the combined stresses of high sugar and ethanol (2).

The boost in tolerance conferred by heightened K^+ and pH extends to higher alcohols capable of serving as unmodified substitutes for gasoline. Although at lower concentrations when compared to ethanol (reflecting their increased

toxicity), we observed that viability is similarly enhanced when cells are shocked using step increases of isopropanol and isobutanol (Fig. 2, D and E, and fig. S10B). That the improvements are not unique to ethanol suggests that these adjustments to the medium augment a more general cellular process involved in alcohol resistance or membrane integrity.

Given the dominant effects exerted by extracellular K^+ and pH, we hypothesized that K^+ and proton (H^+) regulation may be a primary mechanism mediating the tolerance phenotype. Because opposing gradients of K^+ and H^+ ions are generated across the yeast plasma membrane by the K^+ importer *TRK1* and H^+ exporter *PMA1* (18, 19), we surmised that genetic modifications of these adenosine triphosphate-dependent pumps, designed to perturb or strengthen these gradients, would produce corresponding effects to fermentative performance. Indeed, genetic deletions that debilitate K^+ import or H^+ export weakened ethanol output (fig. S11). Likewise, hyperactivation of *TRK1*, accomplished by deletions of *PPZ1* and *PPZ2* (19), generated an increase, yielding an $18 \pm 1.6\%$ (SD) improvement in titer over the wild type when cultured in unmodified medium (Fig. 3 and fig. S12A). Given the electroneutral codependence of the K^+ and H^+ gradients (18), we, furthermore, reasoned that up-regulation of *PMA1* alongside augmented *TRK1* would further enhance the ethanol phenotype; indeed, the combination increased titers by $27 \pm 2.2\%$ over the wild

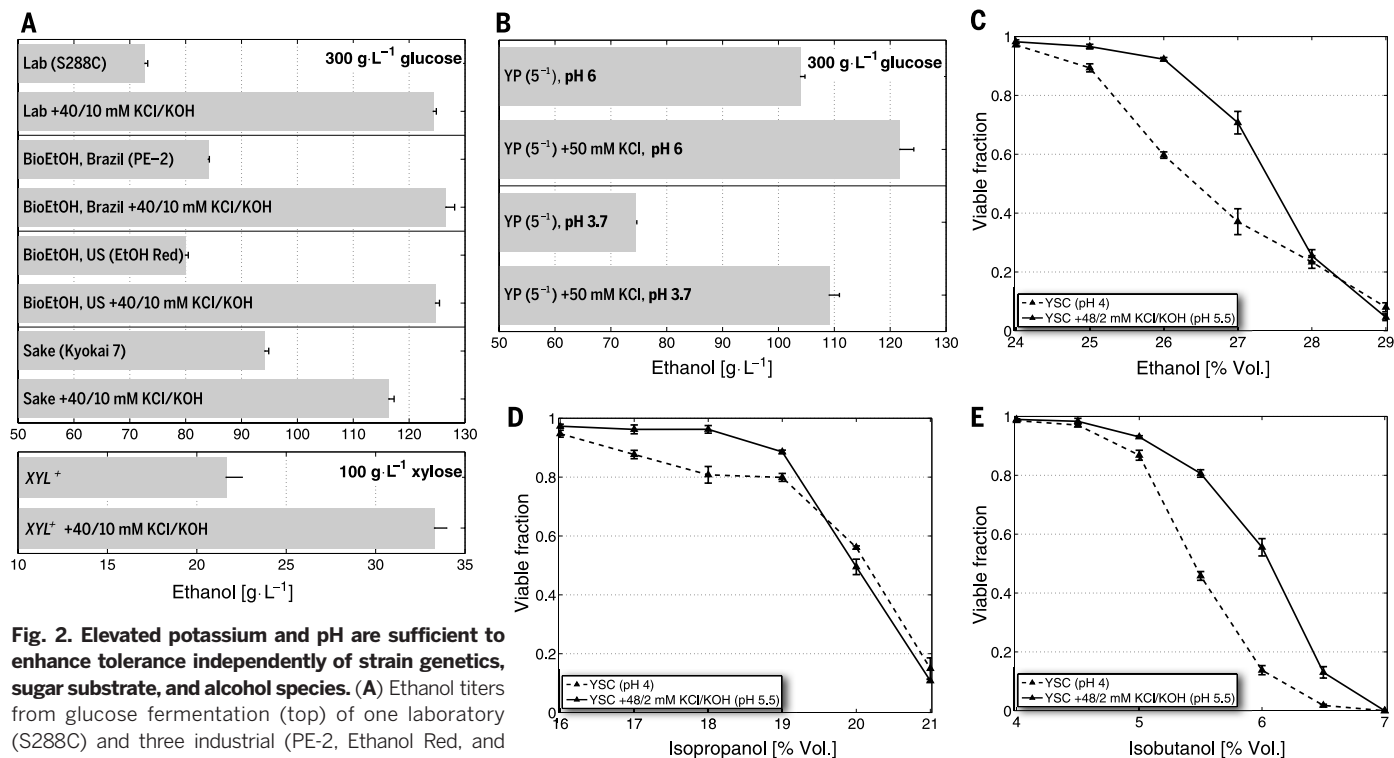


Fig. 2. Elevated potassium and pH are sufficient to enhance tolerance independently of strain genetics, sugar substrate, and alcohol species. (A) Ethanol titers from glucose fermentation (top) of one laboratory (S288C) and three industrial (PE-2, Ethanol Red, and Kyokai 7) yeast strains, or from xylose fermentation (bottom) of an engineered xylose strain, in unmodified YSC or YSC supplemented with 40 mM KCl and 10 mM KOH. (B) Titers from S288C cultured in 20% YP medium or medium supplemented with potassium, at pH 6 and 3.7. (C) Population fractions of S288C after transfer from overnight growth in unmodified YSC (dashed lines) or medium supplemented with 48 mM KCl and 2 mM KOH (solid lines) into media containing the indicated concentrations of ethanol. (D and E) Same as (C), but with step increases of isopropanol or isobutanol, respectively. All data are mean $\pm$ SD from three biological replicates.

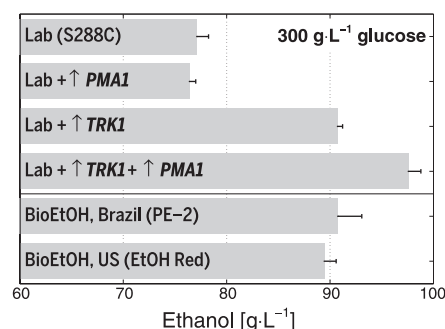


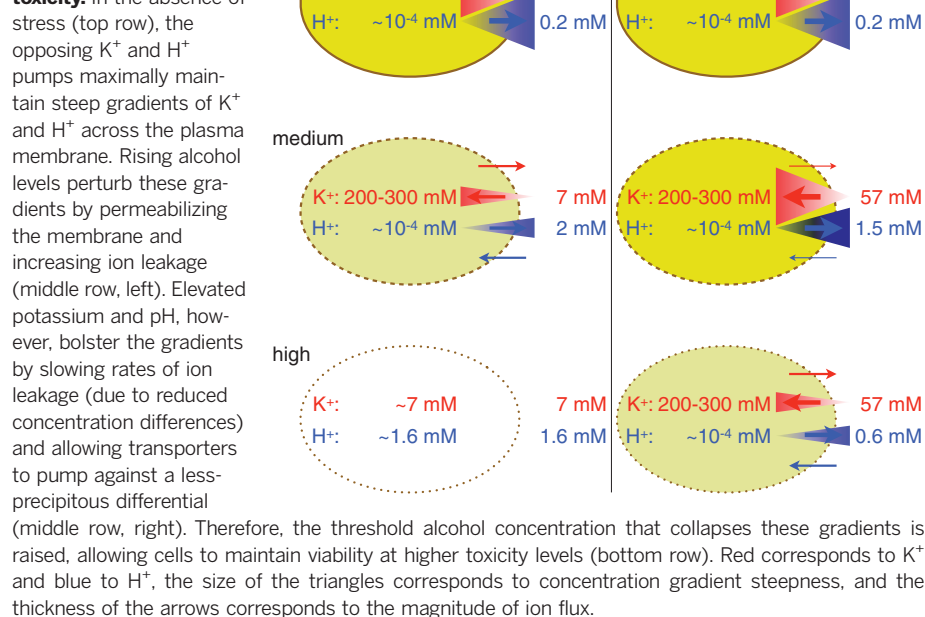
Fig. 3. Genetic augmentation of the plasma membrane potassium (*TRK1*) and proton (*PMA1*) pumps increases ethanol production to levels exceeding those of industrial strains. Shown are ethanol titers from a wild-type laboratory strain (S288C) transformed with empty overexpression plasmid, S288C transformed with a plasmid overexpressing *PMA1*, S288C containing hyperactivated *TRK1* (via deletions of *PPZ1* and *PPZ2*) and transformed with empty overexpression plasmid, the *TRK1* hyperactivated strain transformed with a plasmid overexpressing *PMA1*, and bioethanol production strains from Brazil (PE-2) and the United States (Ethanol Red), all cultured in unmodified YSC lacking uracil. Data are mean $\pm$ SD from three biological replicates.

type. These improvements in output mirrored enhancements in net fermentation viability (Fig. S12B), affirming the coupled nature of production and tolerance. Incidentally, overexpression of *PMA1* without hyperactivation of *TRK1* did not enhance ethanol performance (Fig. 3), supporting the proposed notion that K^+ uptake creates the dominant electromotive force, and H^+ efflux acts primarily as a response current (19).

Genetic augmentation of the K^+ and H^+ transporters increased ethanol titer of the laboratory strain to that surpassing the two bioethanol production strains (Fig. 3). These specific genetic modifications are, therefore, sufficient to create a superior phenotype that was previously available only through selection. That hyperactivation of *TRK1* alone is sufficient to match the titers of PE-2 and Ethanol Red, combined with the observation that these industrial strains respond to KCl and KOH supplementation, lends support to the possibility that polymorphisms enhancing the K^+ and H^+ gradients may be responsible for intrinsically higher ethanol tolerances (20).

Collectively, our results suggest a toxicity model in which alcohols attack viability not at threshold concentrations that solubilize lipid bilayers but at lower concentrations that increase the permeability of the plasma membrane and dissipate the cell's ionic membrane gradients. The observation that genetically unchanged cells can be made to tolerate higher ethanol concentrations by modulating extracellular K^+ and pH indicates that many observed tolerance thresholds (such as the sub-100 g/liter titers from unmodified medium) represent a physiological, rather than chem-

Fig. 4. Potential biophysical mechanism depicting how elevated potassium and pH counteract rising alcohol toxicity. In the absence of stress (top row), the opposing K^+ and H^+ pumps maximally maintain steep gradients of K^+ and H^+ across the plasma membrane. Rising alcohol levels perturb these gradients by permeabilizing the membrane and increasing ion leakage (middle row, left). Elevated potassium and pH, however, bolster the gradients by slowing rates of ion leakage (due to reduced concentration differences) and allowing transporters to pump against a less-precipitous differential (middle row, right). Therefore, the threshold alcohol concentration that collapses these gradients is raised, allowing cells to maintain viability at higher toxicity levels (bottom row). Red corresponds to K^+ and blue to H^+ , the size of the triangles corresponds to concentration gradient steepness, and the thickness of the arrows corresponds to the magnitude of ion flux.



ical, limit. Ethanol has been known to decrease intracellular pH in a dose-dependent fashion, demonstrating that its amphipathicity permeabilizes the plasma membrane to H^+ (and potentially other ions) (8, 21). Furthermore, that the coupled K^+ and H^+ gradients comprise a dominant portion of the yeast electrical membrane potential, used to power many of the cell's exchange processes with the environment, hints that the cessation of nutrient and waste transport due to gradient dissipation may be a primary mode of cell death (18, 21–25).

These ionic gradients are probably disrupted with increasing strength as ethanol accumulates during fermentation, requiring the cell to expend escalating amounts of energy to reestablish the steep separation of charges. Conditions that bolster the cell's efforts to maintain high concentrations of intracellular K^+ and low intracellular H^+ [estimated to be 200 to 300 mM and pH 7, respectively (23, 24)] thus probably enhance tolerance by raising the threshold at which alcohols will collapse these gradients (Fig. 4). Physical reinforcements in the form of K^+ supplementation and acidity reduction generate the greatest improvements: Not only do higher concentrations of extracellular K^+ facilitate import (by potentially reducing the gradient from 36- to 4-fold, using the above estimates) and lower concentrations of extracellular H^+ facilitate export, the corresponding rates of ion leakage are decreased because of reduced concentration differences across the membrane. Likewise, genetic modifications strengthening the ion pump activities responsible for establishing the K^+ and H^+ gradients increase performance without alteration of the medium.

However, that KCl and KOH supplementation confers larger improvements suggests that tolerance may contain a considerable physically driven component that ultimately constrains biological augmentation.

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SUPPLEMENTARY MATERIALS

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 Materials and Methods
 Supplementary Text
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BIOFUELS

Altered sterol composition renders yeast thermotolerant

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Ethanol production for use as a biofuel is mainly achieved through simultaneous saccharification and fermentation by yeast. Operating at $\geq 40^\circ\text{C}$ would be beneficial in terms of increasing efficiency of the process and reducing costs, but yeast does not grow efficiently at those temperatures. We used adaptive laboratory evolution to select yeast strains with improved growth and ethanol production at $\geq 40^\circ\text{C}$. Sequencing of the whole genome, genome-wide gene expression, and metabolic-flux analyses revealed a change in sterol composition, from ergosterol to fecosterol, caused by mutations in the C-5 sterol desaturase gene, and increased expression of genes involved in sterol biosynthesis. Additionally, large chromosome III rearrangements and mutations in genes associated with DNA damage and respiration were found, but contributed less to the thermotolerant phenotype.

Microbial fermentation of biomass-derived feedstocks represents an attractive solution for production of sustainable liquid transportation fuels (1, 2). About 100 billion liters of ethanol are produced annually by fermentation of mainly sugarcane saccharose and corn starch by the yeast *Saccharomyces cerevisiae* (3, 4). There is also a growing interest in using this yeast for production of advanced biofuels with properties more similar to those of petroleum-derived fuels, and for using other biomass for fermentation, such as lignocellulose from residual biomass (2, 5–7). The production of ethanol or advanced biofuels benefits greatly from fermentations at high temperature ($\geq 40^\circ\text{C}$), because this reduces cooling costs and helps prevent contamination (3, 8). High operating temperature also enables more efficient hydrolysis of the feedstock, thus leading to improved productivities in simultaneous saccharification and fermentation (8–10). In these processes, thermophilic enzymes and yeast are added simultaneously to ensure concurrent

hydrolysis of starch or biomass into monosaccharides, and their further conversion to biofuels by yeast fermentation. However, temperatures $\geq 34^\circ\text{C}$ seriously impair yeast viability and growth.

Thermotolerance in yeast *S. cerevisiae* can be induced by short-term exposure to nonlethal stress conditions including low pH, high osmolarity, elevated ethanol concentrations, and superoptimal temperatures ($\geq 37^\circ\text{C}$) (11, 12). The acquired thermal protection is, however, nonheritable and is attributable to induction of cellular responses including the accumulation of heat-shock proteins (Hsp) and/or trehalose, which can provoke cell cycle arrest at the G_1 phase, and low adenosine 3',5'-monophosphate-protein kinase (cAMP-PK) activity concomitant with reduced glycolytic fluxes (11, 12). Thus, adaptation due to the activation of the heat-shock response is not the most suitable strategy for biofuel production. Industrial thermotolerant yeast strains currently used for bioethanol production were selected in the production processes, where they were exposed to high temperatures for longer periods of time (3, 4). These strains can grow and consume glucose faster at higher temperatures than strains that have just been adapted by stress induction. Although the industrial strains have been used globally for ethanol production, little is known about the genomics of the strains and what makes them thermotolerant (4).

We established three independent yeast populations from three individual clones of the haploid wild-type *S. cerevisiae* yeast strain CEN.PK113-7D, and we used adaptive laboratory evolution (ALE)

to select thermotolerant strains that were extensively characterized (13).

Our ALE approach resulted in three parallel selections: Three clonal populations grew at $39.5 \pm 0.3^\circ\text{C}$ for more than 90 days, producing over 300 generations (Fig. 1A). After 326, 344, and 375 generations, the specific growth rate (μ) increased on average 1.57 ± 0.11 times in all three populations. We determined the specific growth rate of nine thermotolerant strains (TTs), three randomly picked from each population (Fig. 1A). Seven TTs had very similar growth kinetics at $40 \pm 0.1^\circ\text{C}$ under fully aerobic conditions (Fig. 1B). These strains grew on average 1.91 ± 0.12 times faster, consumed glucose on average 1.50 ± 0.2 times faster, and excreted ethanol and glycerol on average 1.6 ± 0.09 and 1.3 ± 0.08 times faster, respectively, than the parental strain (Fig. 1, B and C). The biomass yield on glucose increased by an average of $18 \pm 0.5\%$ (Fig. 1D).

We sequenced the genomes of the seven strains (TT11, TT12, TT13, TT21 TT22, TT31, and TT33) and performed whole-genome transcriptional profiling during exponential growth on glucose in bioreactors at $40 \pm 0.1^\circ\text{C}$. Whole-genome sequencing revealed a total of 30 single-nucleotide variations (SNVs) in 18 genes (tables S1 and S2). The average number of SNVs per genome duplication was $1.9 \times 10^{-9} \pm 2.6 \times 10^{-10}$, and the average number of SNVs per 100 generations was 2.43 ± 0.3 , which is higher than that reported for adaptation to other stresses (1.13 to 2.13 per 100 generations) (14). Most SNVs were detected in genes affecting membrane composition and structure, respiration, DNA repair, and replication (Fig. 2A). Nonsense mutations in the C-5 sterol desaturase gene *ERG3* were present in all lineages (table S2). Large genetic duplications were also found in five out of the seven strains. These consisted of two different types of segmental duplications of chromosome III (ChrIII), each found in strains isolated from two of the lineages (Fig. 2, B and C). It was previously reported that complete duplication of ChrIII in the diploid *S. cerevisiae* strains was selected during growth at 39°C , but this reported duplication was unstable and disappeared after 600 generations (15).

The three evolved yeast strains isolated from population 1, TT11, TT12, and TT13 all had a duplicated ChrIII region containing the *HCM1*, *RRT12*, *IMG1*, *IMG2*, *PER1*, and *YCR007* genes (fig. S1). A previous study showed that overexpression of these genes contributed to up to 23.5% of the thermotolerant phenotype (15). We did not find that duplications of some of these genes on ChrIII resulted in higher gene transcription,

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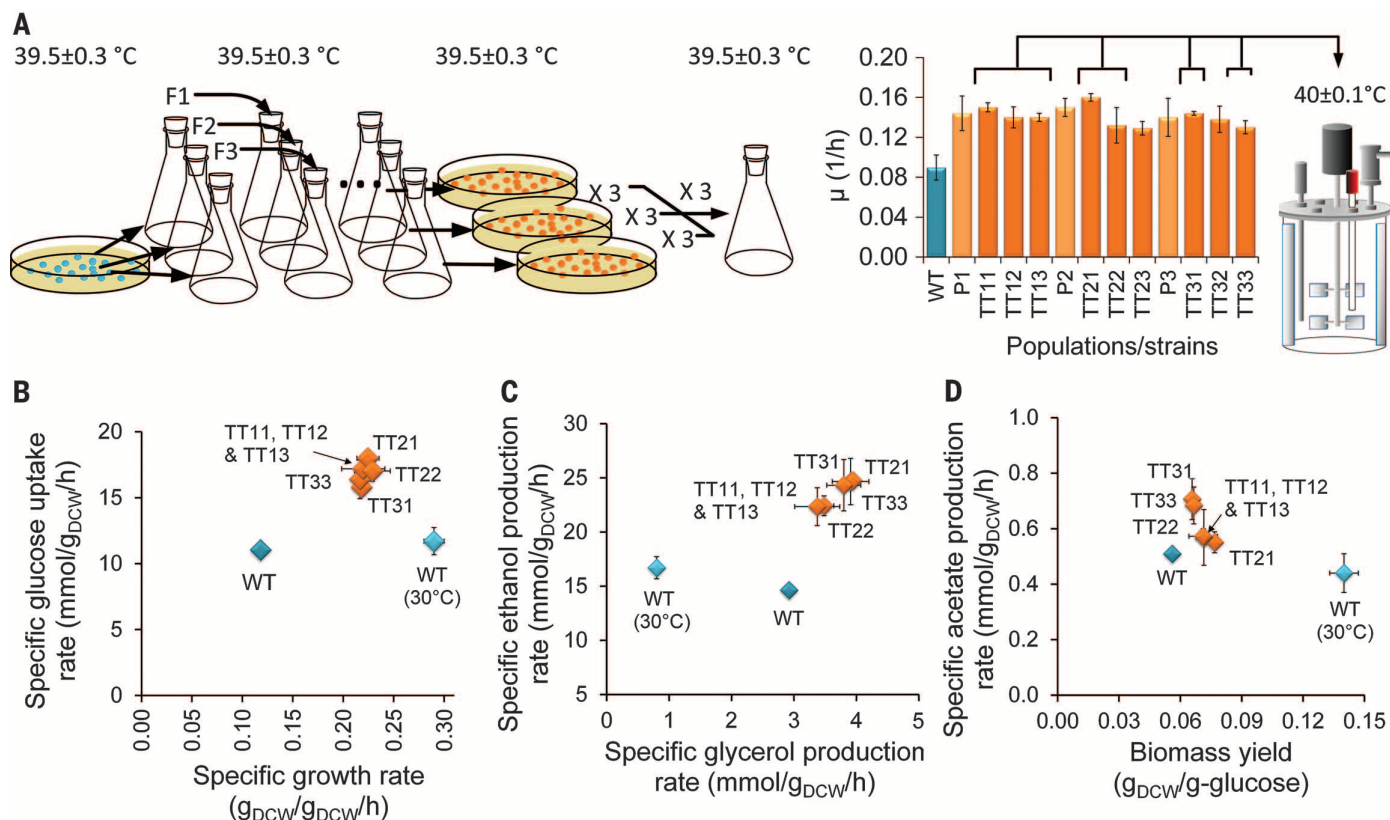


Fig. 1. Adaptive laboratory evolution and physiological characterization of thermotolerant *S. cerevisiae* strains. (A) Experimental setup for selecting thermotolerant *S. cerevisiae* strains using adaptive laboratory evolution in shake flasks. Evolved mutants from flask/population 1 have the same sequence (Fig. 2A). Physiological responses in bioreactors at 40 ± 0.1 °C are plotted in the graphs (B to D). Samples were taken from bioreactors in the middle exponential phase of growth for transcriptomics profiling using microarrays.

as was shown in (15) (table S3). HSP30, which negatively regulates H(+)-ATPase (adenosine triphosphatase), thereby avoiding excessive consumption of ATP during heat shock (16), was duplicated on ChrIII (in TT31 and TT33) but was expressed less than in the control.

HCM1, CDC39, KRE28, SLK19, DYN2, CGR1, LSM8, LSM2, and PAT1—genes that have an important role in M and G₁ phases—were all over-expressed (fig. S1). Products of LSM8, LSM2, PAT1, and PWP2 and RSA4 (ChrIII duplication) perform key tasks in ribosome pre-ribosomal RNA (rRNA) processing and 60S ribosome subunit synthesis (17) and are overexpressed upon DNA damage (18). Furthermore, mutations in genes for cell-cycle check point regulations and DNA damage networks (RAD9, RAD24, and PAN2) (Fig. 2A) could suggest that the DNA damage response may facilitate yeast to grow at super-optimal temperatures. However, TT21 did not accumulate mutations in any of these networks and was as fit at 40 °C as other TT strains.

TUP1 and SRB8 (ChrIII duplication) were over-expressed in TT11, TT12, and TT13 (table S3). The Tup1-Cyc8 co-repressor is important for repression of many genes (19); it also interacts with the transcription machinery, including SRB8. Transcription factors YAP6, CIN5, NRG1, and ROX1 (fig. S1), which are regulated by TUP1 and SRB8 and mediate glucose repression and aerobic transcriptional

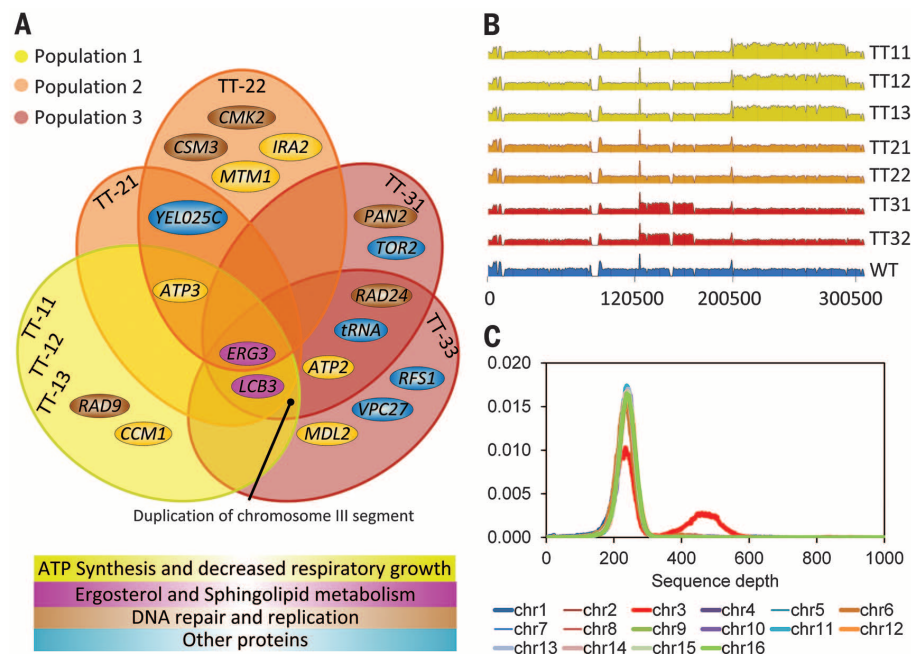


Fig. 2. Specific mutations and duplications of chromosome segments were selected independently during the evolution of three populations of *S. cerevisiae*. (A) Distribution of 19 genes, with 31 SNVs among 7 TTs, encoding proteins involved in three cellular processes. (B) Segmental duplications in chromosomes were identified from gene-coverage analysis. (C) TT11 genome shown as an example.

Fig. 3. Specific growth rate and total sterols composition of selected and reconstructed thermotolerant *S. cerevisiae* strains. (A) Specific growth rate of the selected thermotolerant strain TT11, wild-type strain carrying point-mutation M7 (coding for Erg3^{Tyr185}), wild-type strain carrying point-mutation M22 (coding for Atp3^{Glu299}), and wild-type strain (WT). (B) Sterol analysis was carried out on the same strains. All characterizations were done on strains cultivated in shake-flasks at $39.5^{\circ} \pm 0.3^{\circ}\text{C}$ in minimal medium with glucose. Samples for sterol analysis in strains TT11, M7, and WT were taken at the end of the exponential phase. (C) Fecosterol and ergosterol three-dimensional structures.

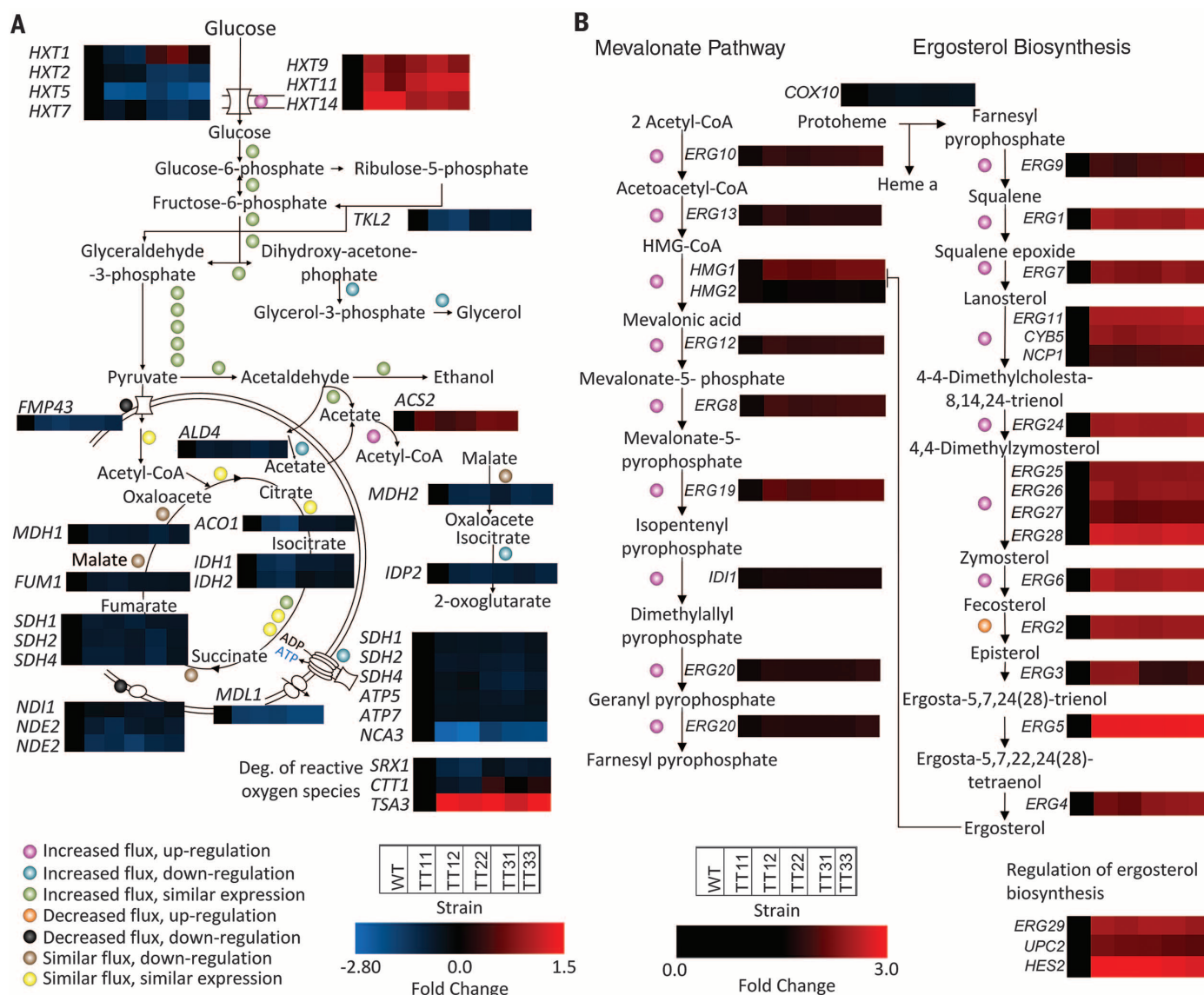
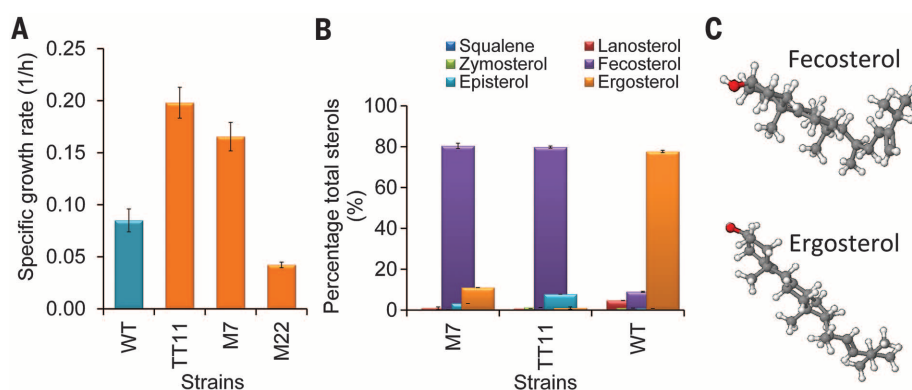


Fig. 4. Changes in gene expression and metabolic fluxes in thermotolerant strains. Transcriptional profiling of TTSs was performed at 40°C in bioreactors and compared to the parental strain in the same conditions. The yeast genome-scale model *iIN800* and external fluxes were used to calculate metabolic fluxes, which were then used in combination with transcriptional profiling to analyze changes in flux using the Random sampling algorithm (additional data tables S1 and S2). (A) Gene transcription and metabolic fluxes of the central metabolism are changed in the selected strains (TTSs) when compared to the WT. (B) Gene transcription and metabolic fluxes of the mevalonate pathway and sterol biosynthesis are up-regulated and have increased flux in the selected strains when compared to the WT.

repression, were down-regulated. Higher tolerance to osmotic stress could be related to the duplication of the mitogen-activated protein kinase (MAPK) gene *SSK22* (part of the Hog1 signaling pathway) in TT11, TT12, and TT13 strains (fig. S2). The MAPK cascade involved in control of mating was down-regulated in TTs together with the cell-cycle factor arrest genes *FAR1* and *STE12* (fig. S2A), which activate genes involved in mating or pseudohyphal and invasive growth pathways, along with the transcription factor *TEC1*. Consequently, TTs had fewer invasive phenotypes (fig. S2C).

Twenty-six percent of SNVs located in coding regions resulted in stop codons (table S2), with 66% of those being in the *ATP3* and *ERG3* genes. *ATP3* codes for the γ subunit of the F_1 complex of the mitochondrial F_1F_0 -ATP synthase, and *ERG3* codes for C-5 sterol desaturase. We reconstructed the point mutations in these genes, in the parental background. The mutation in the ATP synthase gene *ATP3* negatively affected thermotolerance, and the strain grew poorly at 40°C (Fig. 3A). By contrast, the reconstructed *ERG3* mutations (nonsense mutation at position 185, replacing Tyr¹⁸⁵ with a stop codon) conferred to the parental strain up to 86% of the specific growth rate of the TTs (Fig. 3A). Sterols contribute to the fluidity of lipid membranes and lipid rafts, which are important for many cellular processes including cellular sorting, cytoskeleton organization, and mating (20, 27). Membrane fluidity is also affected by the ratio, composition, and structure of sterols that are found in membranes. Thus, regulation of type and amount of sterols has an important modulatory role and may serve as an adaptive response to variations in temperature (22). Branched sterols such as sitosterol and bended sterol-like lipids such as bacteriophanetrol were found to protect membranes of plant cells and *Archaea* from temperature fluctuations and high temperatures (fig. S3) (22). The thermotolerant strains and the strain with the *ERG3* point mutations accumulated total sterols in amounts similar to that accumulated by the wild type (fig. S4). *ERG3* deletions have been reported to cause augmented activity of the sterol methyltransferase Erg6 that catalyzes the conversion of zymosterol to fecosterol (23, 24), and we found that the TTs and the *ERG3* reconstructed mutant accumulated the “bended” sterol, fecosterol (Fig. 3C and fig. S3A). Previously, ergosterol substitution by other “flat” sterols such as 8(9),22-ergostadiene-3 β -ol or 5,7,22,24(28)-ergostetraene-3 β -ol did not increase thermotolerance in *S. cerevisiae* (24). This suggests that increased presence of “bended” sterols (as opposed to “flat” sterols that are normally found in membranes) could confer thermotolerance in the evolved *S. cerevisiae* strains.

Lack of ergosterol (due to the *ERG3* mutations) in the evolved strains induced overexpression of genes involved in sterol biosynthesis from acetyl-coenzyme A (CoA)—i.e., the gene encoding cytosolic acetyl-CoA synthetase *ACS2*, key genes of the mevalonate pathway (*ERG10* and *HMG1*), and many genes involved in sterol biosynthesis (Fig. 4). There was also increased expression of *UPC2*, which codes for a sterol regulatory element-binding pro-

tein (SREBP) that regulates expression of genes coding for enzymes involved in sterol biosynthesis. Despite the up-regulation of these genes, the sterol content in TTs was not changed compared to the parental strain (fig. S4).

The thermotolerant yeast strains did not metabolize nonfermentable carbon sources and did not show diauxic shift at 40° or 30°C (fig. S5). All evolved strains accumulated mutations in either *ATP2* or *ATP3* genes that are essential for growth on nonfermentable carbon sources (Fig. 2A and table S2). This suggests that the optimal thermotolerant phenotype could not arise through evolution while oxidative respiration is fully functional, possibly because it would generate more reactive oxygen species (ROS) and induce oxidative stress. Indeed, the thermotolerant strains have lower resistance to oxidative stress induced with H₂O₂, compared to the parental strain (fig. S6). It has been reported that thermotolerant yeasts are facultative anaerobes susceptible to form respiratory-deficient mutants, in contrast to the psychrophilic yeasts that are strict aerobes (25). Indeed, thermotolerant *S. cerevisiae* strains generated by short-term exposure to 54°C or adaptation to grow at 40°C also acquired a respiratory-deficient phenotype concomitant with lack of growing on nonfermentable carbon sources (26, 27). The inability to grow on nonfermentative carbon sources in the temperature-tolerant yeast strains is an undesirable trade-off, as this increases the costs associated with propagation of yeast during the industrial process. However, the strain with the reconstructed point mutation in the *ERG3* gene, which has a thermotolerant phenotype, has maintained the ability to grow on nonfermentable carbon sources and shows a typical diauxic growth (fig. S7).

Despite the inability of the TTs to grow on nonfermentable carbon sources, they all had a ~30% increased oxygen uptake rate and increased flux through the respiratory chain (fig. S8). Metabolic flux analysis shows that these strains have residual tricarboxylic acid cycle activity, and the increased oxygen consumption is solely used for oxidation of the cytosolic NADH (nicotinamide adenine dinucleotide, reduced) generated by the increased biomass yield. Consequently, the TTs have a reduced glycerol yield (fig. S8).

The TTs have an improved specific glucose consumption rate, which has increased by 60% at 40°C and by 300% at 42°C when compared to the parental strain at 40°C or 42°C. Interestingly, TTs could consume glucose at temperatures as high as 50°C. The increased glucose uptake rate and the increased ethanol production and yield (fig. S8) result in higher ATP production that supports faster growth. Glucose consumption rate is controlled by transcriptional regulation of glucose transporter genes (*HXT*) (28). *MTH1* interacts with the transcription factor *RGT1* and thereby causes transcriptional repression of the *HXT* genes (29). *MTH1* was down-regulated in TTs, suggesting less repression of the *HXT* genes, which was consistent with increased expression of the putative hexose transporters Hxt9, Hxt11, and Hxt14. Expression of glycolytic genes was not changed in in TTs cultivated at 40°C (Fig. 4A).

Our multilayer genome-scale characterization of yeast strains revealed several mechanisms needed to acquire thermotolerance, involving cell cycle, DNA replication stress, and altered sterol metabolism. The latter was the most important, as it appeared in all the isolated strains from three independent populations. Because sterols have an important role in regulation of membrane fluidity, allowing vital process such as vesicular sorting and transport, cytoskeleton organization, and asymmetric growth, we hypothesize that these functions are mainly impaired at high temperature. Substitution of “flat” ergosterol by the “bended” fecosterol in evolved yeast strains may be responsible for the optimal membrane fluidity, as occurs in thermophiles and hyperthermophiles (30).

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S8
Tables S1 to S4
References (31–39)
Databases S1 and S2

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MAMMALIAN ENERGETICS

Flexible energetics of cheetah hunting strategies provide resistance against kleptoparasitism

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Population viability is driven by individual survival, which in turn depends on individuals balancing energy budgets. As carnivores may function close to maximum sustained power outputs, decreased food availability or increased activity may render some populations energetically vulnerable. Prey theft may compromise energetic budgets of mesopredators, such as cheetahs and wild dogs, which are susceptible to competition from larger carnivores. We show that daily energy expenditure (DEE) of cheetahs was similar to size-based predictions and positively related to distance traveled. Theft at 25% only requires cheetahs to hunt for an extra 1.1 hour per day, increasing DEE by just 12%. Therefore, not all mesopredators are energetically constrained by direct competition. Other factors that increase DEE, such as those that increase travel, may be more important for population viability.

The acquisition and expenditure of energy by animals unifies physiology with population ecology and viability, although interactions between energetics, ecology, and survival can be complex (1, 2). Indeed, of the studies that have investigated how energetic factors affect population dynamics, most are concerned with the effects of changes in abiotic conditions such as ambient temperature (3), with few examining the effects of changes in biotic conditions, such as the abundance and distribution of prey and competitors (1, 4).

Although recent human activities have driven declines in large mammalian predators (5), intraguild interactions may also shape carnivore communities. One persistent hypothesis suggests that, because carnivores may be routinely working close to maximum sustained power outputs, decreases in food availability or increases in activity may push them over an energetic precipice

(6). Kleptoparasitism, the theft of prey captured by another animal, is one critical element in this interaction, particularly for mesopredators such as wild dogs *Lycan pictus* and cheetahs *Acinonyx jubatus*, which are prone to competition with and displacement by larger, more dominant carnivores such as lions *Panthera leo*, and spotted hyaenas *Crocuta crocuta* (7–11). The details of such intraguild interactions with respect to energetic implications are, however, poorly understood.

Carnivores hunt using a combination of sit-and-wait, stalk, ambush-and-charge, or extended courting strategies (12–15). Although the short-term energetic consequences of hunting (i.e., the ways that predators chase and subdue prey) are profoundly different (2, 16), the long-term costs, such as the energy required to locate prey and avoid predators, are rarely considered. These costs may be pivotal in determining the viability of different hunting strategies, particularly as it relates to prey abundance, accessibility, and loss (2, 6, 17).

We combined behavioral observations of 14 cheetahs from the Kgalagadi Transfrontier Park (Kalahari) with measurements of daily energy expenditure (DEE) to estimate the energetic cost of foraging. We also obtained DEE measurements of five free-ranging cheetahs from Karongwe Game Reserve (Karongwe). The cheetah is an appropriate study species because it is regarded as vulnerable to kleptoparasitism (8, 9, 18) and has the highest power per given body mass (W/kg) of any mammal during short periods of pursuit (19). This leads to the perception that they experience overall high sustained energetic costs (7). Over 2-week periods, we measured cheetah DEE using the doubly labeled water (DLW) technique (20) while following the animals most days. Various behaviors were recorded (e.g., lying, sitting, walking, and chasing prey), and scat samples were collected periodically. We examine the relationship between DEE and the “prey location” and “prey pursuit” phases of hunts and how this affects their vulnerability to kleptoparasitism. We calculated DEE using isotope analysis of water extracted from multiple excreta samples to provide one measurement of DEE per individual over the 2-week period [multisample DEE (MS-DEE)] as well as on a per diem basis using pairs of samples collected consecutively, providing several measurements of DEE per animal within the period [sequential sample DEE (SS-DEE)]. Means are presented ± 1 SD. For full methodological details, see the supplementary materials.

Mean MS-DEE (8883 ± 3854 kJ/d, $N = 19$ cheetahs) was not significantly different from predictions for free-ranging mammals of similar size (Table 1). The values of sustained metabolic scope (SusMS)—a measure of work rate independent of body size (21) [$1.55 \pm 0.69 \times$ resting metabolic rate (RMR)]—were also not significantly different from allometric predictions (Table 1). There were no study-site or sex-related differences in MS-DEE ($\chi^2 = 0.234$, $P = 0.629$ and $\chi^2 = 0.209$, $P = 0.647$, respectively). Cheetahs were mobile for 2.86 ± 0.95 hours (12%) per day moving at an average speed of 0.83 ± 0.54 m/s (excluding prey pursuits) and chased prey 1.2 ± 0.49 times per day for an average of 37.9 ± 11.6 s per chase.

There were significant intra- and interindividual differences in SS-DEE for each cheetah

Table 1. Means and SDs of body mass (kg), DEE (kJ/d), predicted DEE (kJ/d), SusMS, and predicted SusMS for cheetahs from Karongwe and the Kalahari (31–34). MS-DEE was calculated using the multisample approach of estimating CO₂ production (30). *Significant difference between predicted and measured values at $P < 0.05$.

	Karongwe ($n = 5$ cheetahs)		Kalahari ($n = 14$ cheetahs)	
	Mean	SD	Mean	SD
Mass (kg)	41	5.6	34	4.1
MS-DEE (kJ/d)	9006	3879	8839	3991
Predicted DEE (31)	7942	499	7050	563
Predicted DEE (32)	8106	530	7162	596
Predicted DEE (33)	12,563	755	11,212*	853
SusMS	1.37	0.55	1.61	0.74
Predicted SusMS (34)	1.44	0.02	1.47	0.02

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followed ($F_{18,62} = 1.83$, $P = 0.041$) (Fig. 1). For predators, with a tendency toward a feast or famine feeding regime, this variation in DEE is expected; individuals are likely to skip hunting on days after kills of large prey (2). Cheetahs

were observed to capture prey on 52% of days, and for 65% of those “successful” days, did not capture anything the next day. However, this was not significantly different from the expected capture rate ($\chi^2 = 1.47$, $P = 0.225$) and therefore

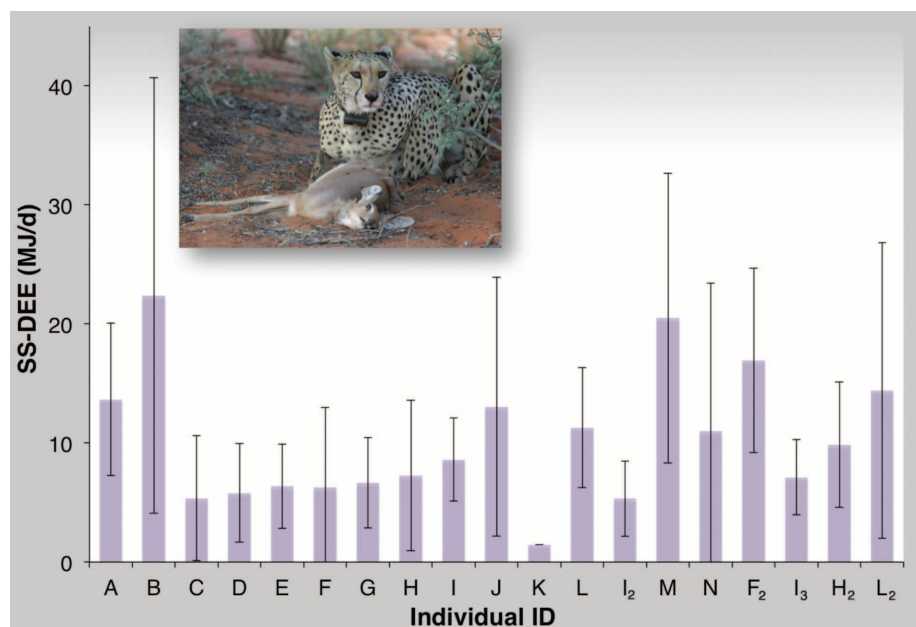


Fig. 1. DEE of cheetahs. Mean energy expenditures (SS-DEE, kJ/d) for 19 measurements, calculated using the two-point method to estimate CO_2 production. Animals A to E were from Karongwe; animals F to L were from the Kalahari. Subscripts indicate repeated measurements within individuals. The order left to right reflects the date of measurement. Error bars denote standard deviations of daily SS-DEE measurements over 2-week periods.

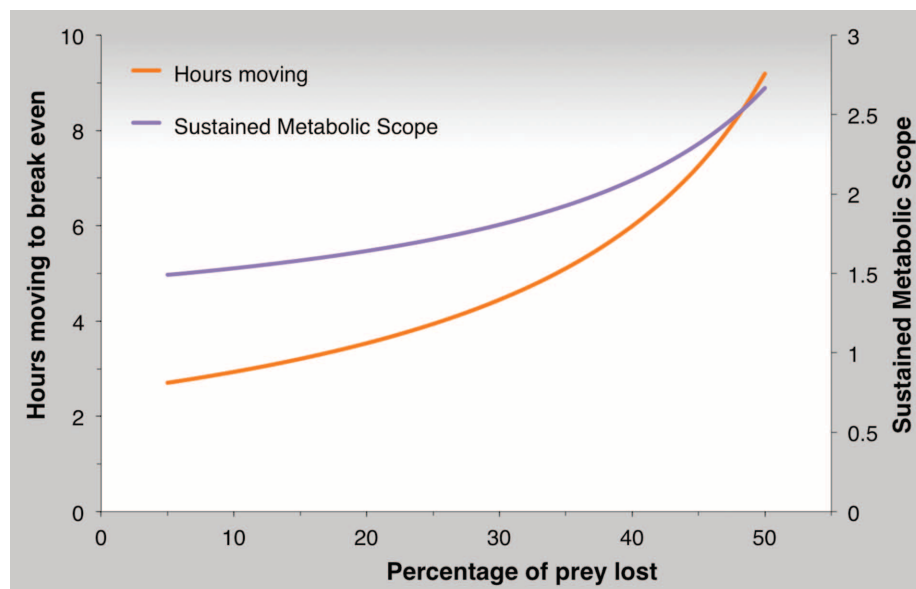


Fig. 2. Model of hours moving to break even and energy balance in cheetahs under different levels of kleptoparasitism. The black line denotes hours spent moving and the red line SusMS (DEE/RMR). The bioenergetic model (6) predicts that if cheetahs lost 25% of their prey to rival predators, they would have to be mobile for 4.0 hours per day to balance energetic needs. Assuming that the costs of moving remain the same, this would elevate DEE during active periods to 5.1 MJ per day or increase total DEE to 10.0 MJ per day (SusMS = $1.7 \times \text{RMR}$). At higher kleptoparasitism rates of 35%, 5.1 hours would be required to be spent mobile (SusMS = $2.0 \times \text{RMR}$), and at 50%, 9.2 hours would be required (SusMS = $2.7 \times \text{RMR}$).

does not provide direct evidence for less hunting after kills. This crude analysis, though, does not factor in how much the animals eat at each kill. There was a positive relationship between distance traveled and the mass of prey eaten on a particular day ($F_{1,46} = 5.98$, $P = 0.018$) and a negative relationship between the mass of prey eaten and the distance traveled the next day ($F_{1,19} = 7.21$, $P = 0.015$), indicating that cheetahs travel less after eating more and when they travel less they have lower intake. A positive relationship also exists between the energy costs of foraging and the perceived risk of predation or interference by predators (22). Consequently, the large variation in MS-DEE observed indicates that cheetahs are capable of operating at high sustained energy expenditures when necessary, whereas the large daily variation in SS-DEE is likely to be driven by variation in activity as a result of differences in feeding success (2), and/or the avoidance of competitors (8, 9, 18).

Importantly, we observed a significant positive relationship between the travel distance on a particular day and SS-DEE ($\chi^2 = 6.36$, $P = 0.012$) but not between pursuit distance and SS-DEE ($\chi^2 = 0.024$, $P = 0.878$). SS-DEE was related to distance traveled by the relationship $\text{DEE (kJ/day)} = 447 \times \text{distance (km)} + 7103$ (least-squares regression, $F_{1,52} = 5.978$, $P = 0.018$, $r^2 = 0.103$). There was also a significant positive relationship between the travel distance on a given day and the distance prey were chased on that day ($F_{1,49} = 5.920$, $P = 0.019$, $r^2 = 0.108$). In terms of daily variation in SS-DEE, we found no evidence that DEE was reduced after days with greater than average DEE ($\chi^2 = 1.60$, $P = 0.206$), although DEE was greater after days with less than average DEE ($\chi^2 = 5.33$, $P = 0.021$). Similarly, cheetahs did not travel further after days of less than average distance moved ($\chi^2 = 3.27$, $P = 0.071$), although they traveled less after days with greater than average distanced moved ($\chi^2 = 5.44$, $P = 0.020$). Because travel distance was the main driver of DEE, any increase, such as might be caused by the need for extra hunting to compensate for kleptoparasitism, will also increase DEE. Kalahari cheetahs were mobile for 12% of the day, which accounted for 42% of the 8.84 MJ total DEE, because being mobile was 5.4 times as costly as resting. The positive relationship between travel distance and pursuit distance may be because increased movement provides additional opportunities for hunting, as observed here, and in Kalahari leopards *Panthera pardus* (23).

Using African wild dogs as an example, Gorman *et al.* (6) suggested that kleptoparasitism affects the population viability of mesopredators. They suggested that activity budgets could be separated into energetically expensive hunting and resting. Escalating losses of prey through kleptoparasitism necessarily increased the time and energy required for hunting, rapidly creating an untenable situation. Kalahari cheetahs are also subject to kleptoparasitism: Of the 43 observed cheetah kills, four (9.3%) were kleptoparasitized (two by brown hyaenas *Hyaena brunnea* and two by lions). Although losing kills increases

the time required to hunt (Fig. 2), our model suggests that, unlike wild dogs, cheetahs are able to cope with kleptoparasitism rates of 25%, because this would only require an additional 1.1 hour per day (a 38% increase) in time spent mobile and increase DEE to 10.0 MJ per day (a 12% increase). Wild dogs may be exceptional in this regard because the high power costs ($25 \times \text{RMR}$, 35 W/kg) and long durations of prey pursuits (3.5 hours per day) make their hunting strategy extremely costly. This contrasts with the hunting strategy of cheetahs; even though power use during pursuit may reach 120 W/kg (19), prey pursuit takes only a few seconds and constitutes a small component of the daily energy budget (undetectable here using doubly labeled water).

Recorded rates of kleptoparasitism in cheetahs are lower than the untenable threshold of over 50% (Fig. 2): 14% in Kruger National Park (24), 11% in the Serengeti (25), and 9.3% in the Kgalagadi Transfrontier Park (current study). Relatively low kleptoparasitism rates in cheetahs that do not change greatly between ecosystems may be due to effective competitor avoidance strategies (9) and a diurnal hunting strategy (26). The comparatively low cost of food acquisition and flexible energy budget of cheetahs compared with that of wild dogs (6) are likely to provide a buffer against varying ecological conditions.

This study lends support to suggestions that interspecific competition does not necessarily suppress cheetah populations (27–30). Instead, it shows that cheetahs are well adapted to the presence of competitors and that costs incurred by traveling drive their energy budgets, rather than those encountered securing prey. Human activities that force cheetahs to travel large distances to avoid disturbance and persecution may push DEE to the limit and consequently compromise their population viability.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/346/6205/79/suppl/DC1
Materials and Methods
Figs. S1 and S2
Table S1
References (35–53)

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MAMMALIAN ENERGETICS

Instantaneous energetics of puma kills reveal advantage of felid sneak attacks

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Pumas (*Puma concolor*) live in diverse, often rugged, complex habitats. The energy they expend for hunting must account for this complexity but is difficult to measure for this and other large, cryptic carnivores. We developed and deployed a physiological SMART (species movement, acceleration, and radio tracking) collar that used accelerometry to continuously monitor energetics, movements, and behavior of free-ranging pumas. This felid species displayed marked individuality in predatory activities, ranging from low-cost sit-and-wait behaviors to constant movements with energetic costs averaging 2.3 times those predicted for running mammals. Pumas reduce these costs by remaining cryptic and precisely matching maximum pouncing force (overall dynamic body acceleration = 5.3 to 16.1g) to prey size. Such instantaneous energetics help to explain why most felids stalk and pounce, and their analysis represents a powerful approach for accurately forecasting resource demands required for survival by large, mobile predators.

A central tenet of foraging theory is that animals manage energetic costs and benefits when feeding (1). Yet measuring these costs for large, highly active predators that hunt, chase, and kill mobile prey has been exceedingly difficult, resulting in a poor understanding of how physiological capacities and environmental factors affect foraging success (2).

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This is especially apparent for species within the family *Felidae*. Among terrestrial carnivores, felids show a large range in body size, prey preferences, and predatory movements, each of which are linked to the landscape in which they live (3–6). The lankiest cat, the African cheetah (*Acinonyx jubatus*), engages in astonishing high-speed pursuits in open habitats to outmaneuver and overtake smaller, swift prey (7, 8). In contrast, heavy-bodied species including African lions [*Panthera leo* (9)], leopards (*Panthera pardus*), tigers (*Panthera tigris*), and pumas residing in forested or grassland habitats tend to stalk, ambush, and pounce to overpower prey up to several times their size (6).

Of the 36 extant wild felid species, the majority are considered cryptic ambush hunters

(5, 6), which would suggest an energetic advantage for this predatory tactic (1). However, because of the covert nature of these activities, it is difficult to observe felid ambush behaviors or measure energetic efficiency. By necessity, field energetic studies of most terrestrial carnivores have relied on broad approaches, including doubly labeled water methods and field metabolic rate (FMR) modeling that integrate energetics across days or seasons [e.g., (10–12)].

Here, we monitored behavior-specific energetics of a large cryptic felid, the puma, to evaluate the cost of discrete physiological states involved with ambush hunting. Using a laboratory-to-field approach, we developed and calibrated a new SMART (species movement, acceleration, and radio tracking) wildlife collar on captive, adult pumas ($n = 3$), with subsequent deployment on wild pumas ($n = 4$ pumas with SMART collars, 1 puma with GPS only; tables S1 and S2). We created a library of behavior-specific collar acceleration signatures by filming instrumented, trained pumas as they traversed a 20-m level course at different speeds, performed routine activities (resting, eating, grooming, etc.), and ran on a motorized treadmill (13). Accelerometer signatures were then correlated to energetic costs by simultaneously measuring oxygen consumption ($\dot{V}_{O_2}$), kinematics [stride frequency, stride length (14)], and overall dynamic body acceleration [ODBA (2, 15)] of the pumas during steady-state resting and treadmill running. Cost of transport (COT, the energy expended per meter) and cost of acceleration (COA, the energy ex-

pended per g) were calculated by dividing $\dot{V}_{O_2}$ by speed and ODBA, respectively.

In the field, we captured and instrumented wild pumas ($n = 2$ females, 2 males) with the calibrated SMART collar. An additional female instrumented with a GPS collar allowed for a detailed analysis of hunting movements and longer deployment due to the conservation of collar batteries. All pumas were then released into a 1700-km² study area within the Santa Cruz Mountains of California. The collar integrated a three-axis accelerometer and magnetometer that continuously monitored activity level and body position at 64 Hz, with a GPS/VHF unit with remote download capabilities that sampled every 4 hours to provide movement and kill locations (13). Potential kill sites were initially identified by the clustering of GPS positions indicating a feeding event, using a custom program integrated in ArcGIS (v.10; ESRI, 2010). Carcass visitation based on the GPS data allowed positive prey identification (13, 29). The timing of the GPS cluster was then correlated to time-synched accelerometer signals and behavioral signatures that indicated a pouncing event. Together, the suite of data from the collar allowed us to map both physiological and behavioral responses of wild pumas onto the physical landscape in which they hunted.

Our results indicate that the biomechanics and energetics of running by pumas are typical of most quadrupedal mammals including other adult felids (16). Both stride frequency and stride length of pumas increased linearly with running speed (Fig. 1A). Pumas also switched among

walking, trotting, and running gaits at transition speeds predicted for their body mass (17). Routine preferred speed on the outdoor course and treadmill was 1.1 m s⁻¹; maximum speed (4.9 m s⁻¹) was only one-fifth of that measured for cheetahs chasing prey (7, 8).

The oxygen consumption (ml O₂ kg⁻¹ min⁻¹) of pumas increased linearly with running speed (m s⁻¹; Fig. 1B) according to

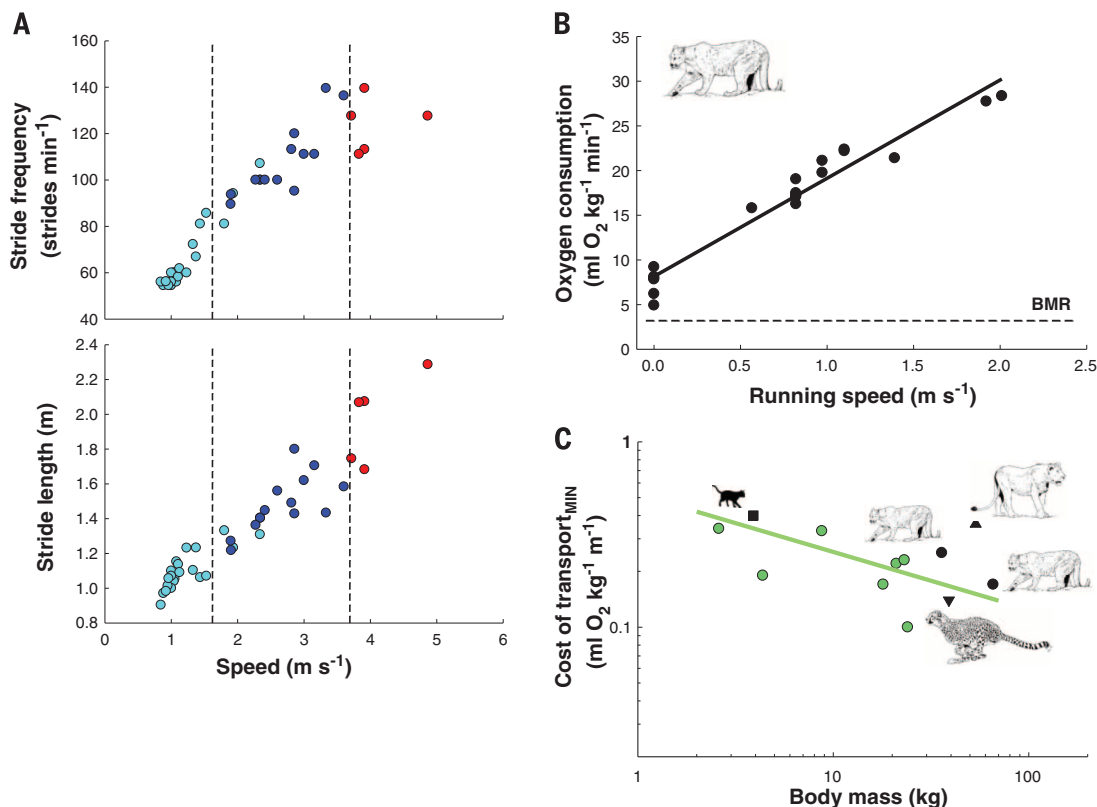
$$\dot{V}_{O_2} = 8.15 + 10.99(\text{speed})$$

$$(r^2 = 0.95, n = 20, P < 0.0001) \quad (1)$$

Minimum cost of transport (COT_{MIN}) was 0.17 ml O₂ kg⁻¹ m⁻¹ (3.42 J kg⁻¹ m⁻¹), as predicted for canids and other felids on the basis of body mass (Fig. 1C) (16). This includes large and small felids that stalk, pounce, and perform high-speed chases. Immature African lions, however, are outliers with a COT_{MIN} that is 2.4 times that predicted for running mammals (18) and 2.1 times the value for pumas. If adults follow the trend for immature lions, then comparatively high locomotory costs of African lions may help to explain the tendency of this species to rely on cooperative hunting, which is unique among felids (6).

Continuous monitoring of acceleration in wild pumas wearing SMART collars allowed us to determine how these costs vary with hunting across time and space for individuals. Figure 2A shows a typical GPS track on the day of a kill for a 42-kg female puma hunting black-tailed deer (*Odocoileus hemionus*). The cat moved downhill through a residential area, stalked and killed

Fig. 1. Stride mechanics and energetic costs of running pumas. (A) Stride frequency and stride length in relation to outdoor speed ($n = 3$ pumas). Points represent individual walk (cyan), trot (blue), or run (red) trials. Dashed lines show predicted gait transition speeds (17). (B) Oxygen consumption in relation to speed for pumas on a treadmill. Points represent individual steady-state measurements. The least-squares regression (solid line, Eq. 1) is compared to basal metabolic rate [BMR, dashed line; (25)]. (C) Minimum cost of transport (COT) for canids [green circles (16)], pumas [black circles, present study and (30)], and other felids [domestic cat, black square; African lion, upward triangle; African cheetah, downward triangle (16)]. The green line is the allometric regression for COT_{MIN} of quadrupeds (16).



a deer, and stayed within 200 m of the carcass until moving into the mountains the next day. What is not apparent in such GPS tracks is the considerable variation in behaviors and energy expended by a puma when pursuing different-sized prey. Here the continuous, high-resolution time-stamped accelerometer traces from the SMART collar provide remarkable insight. First, unique accelerometer signatures within the traces reveal individual behaviors that can be localized via GPS on the terrain where they occur (Fig. 2A). Second, raw triaxial acceleration traces (Fig. 2A, bottom) can be collapsed into overall dynamic body acceleration (ODBA) values and then transformed into activity-specific metabolic costs in terms of $\dot{V}_{O_2}$ ($\text{ml O}_2 \text{ kg}^{-1} \text{ min}^{-1}$) using the regression

$$\dot{V}_{O_2} = 3.52 + 58.42(\text{ODBA})$$

$$(r^2 = 0.97, n = 9, P < 0.0001) \quad (2)$$

from the instrumented pumas walking on a treadmill (Fig. 2B) (13).

Equation 2 allows the acceleration signatures of wild pumas to be assigned to the energetic cost associated with each behavior. As reported for smaller animals and humans (15, 19), pumas

show a significant, linear correlation between oxygen consumption and ODBA. Like COT (16), COA declines asymptotically with ODBA (Fig. 2B, inset), showing a decline in the variation of acceleration costs as activity level increases (i.e., <10% change in COA with ODBA from 0.5 to 3.0 g). The lowest COA ($\sim 58 \text{ ml O}_2 \text{ kg}^{-1} \text{ min}^{-1}/\text{g}$ ODBA) occurs at the highest ODBA and is equivalent to the slope of the relationship in Eq. 2. Together, these relationships enable energetic costs to be ascribed to a wide variety of aerobic activities as long as ODBA accurately reflects the puma's movements (2, 15, 19).

Using these methods, we assigned energetic costs for pumas during three characteristic periods: (i) pre-kill activity (locating prey), (ii) pounce and kill, and (iii) post-kill prey handling and eating. These periods constitute a characteristic 2-hour interval we term the "hunt" (Fig. 2 and Fig. 3). Activity levels during the pre-kill phase varied markedly among individuals, ranging from activities with low energetic cost [sit-and-wait (male puma 5M) and slow-walking (female 2F) behaviors] to those with moderate cost [couraging-type movements (16M) and stalk-and-ambush activities (7F)] (Fig. 3). Total energy expended during the pre-kill period (Hunting Cost_{PRE-KILL} in kJ)

decreased with increased use of cryptic behaviors according to

$$\text{Hunting Cost}_{\text{PRE-KILL}} = 3516.0 - 24.9$$

$$(\% \text{time cryptic})$$

$$(r^2 = 0.99, n = 6 \text{ kills}, P < 0.0001) \quad (3)$$

(Fig. 4A). Here, percent time cryptic represents the proportion of the pre-kill period comprising all low-acceleration, low-cost activities [including rest, slow-walking (e.g., stalking), and sit-and-wait behaviors]. Overall, pre-kill hunting costs represented 10 to 20% of the estimated FMR of pumas (13).

Pumas generally remained aerobic during the hunt, only briefly exceeding their predicted $\dot{V}_{O_{2\text{max}}}$ [49.2 to 54.7 $\text{ml O}_2 \text{ kg}^{-1} \text{ min}^{-1}$ (20)] when pouncing. This high-energy activity, along with killing and prey handling, resulted in different acceleration signatures for pumas preying on fawns or bucks (Fig. 3). Predator costs (kJ) are related to prey mass (kg) as

$$\text{Energetic cost of pounce and kill} =$$

$$88.94 - 5.53(\text{prey mass})$$

$$(r^2 = 0.81, n = 5 \text{ known kills}, P = 0.036) \quad (4)$$

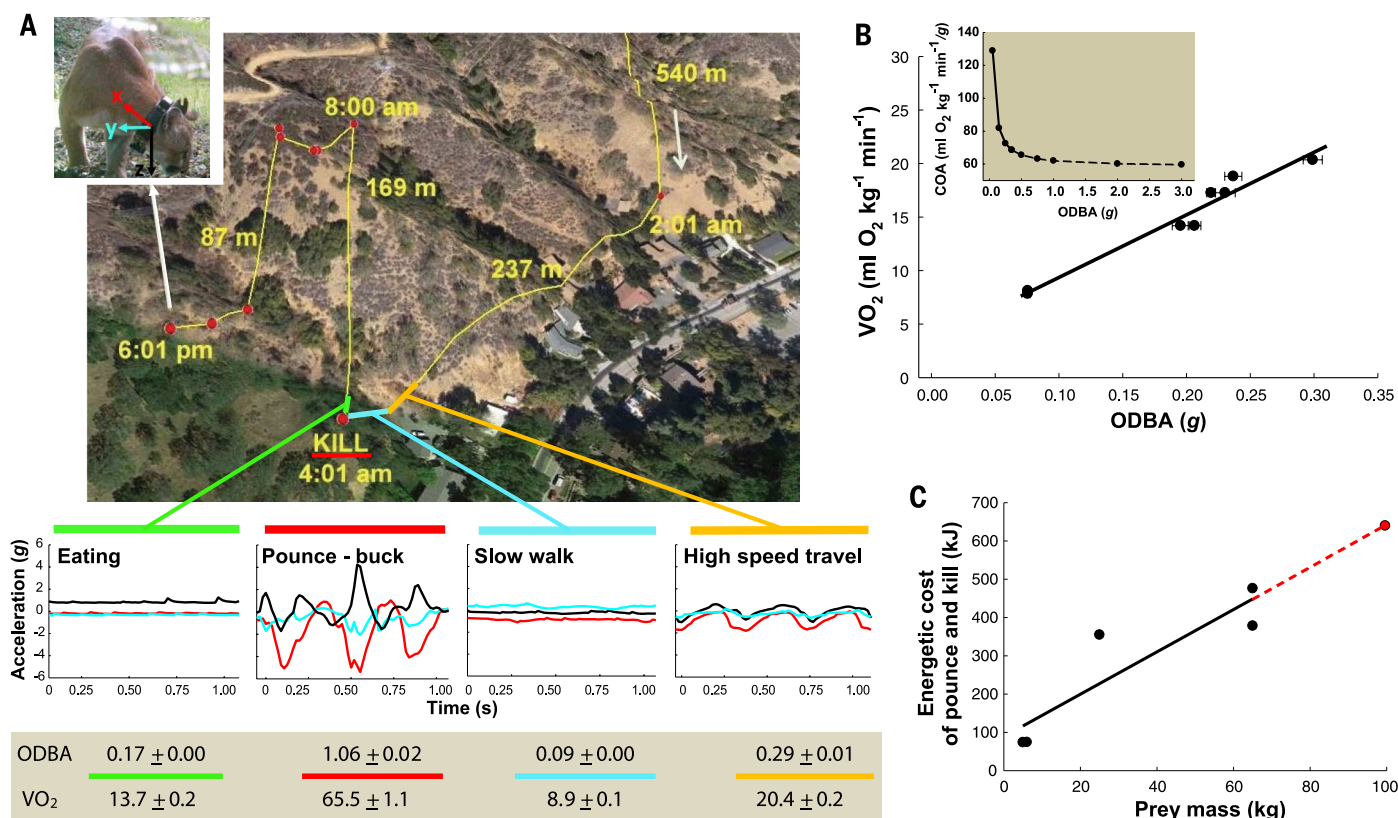


Fig. 2. Field movements, acceleration, and energetic costs for pumas. (A) GPS track for female 13F preying on deer (Google Earth, earth.google.com). Minimum distances moved, time of day, and kill time are indicated. Locations of behavior-specific acceleration signatures ($n = 3840$) (X-surge, red; Y-sway, cyan; Z-heave, black; from collar orientation, inset) are superimposed on the GPS track with corresponding color-coded ODBA (g) and $\dot{V}_{O_2}$ ($\text{ml O}_2 \text{ kg}^{-1} \text{ min}^{-1}$). (B) Relationship between $\dot{V}_{O_2}$ and ODBA for instrumented pumas on a treadmill.

Points are mean steady-state measurements ± 1 SE for two pumas. See text for regression (black line) and COA (inset) statistics. (C) Energetic cost of a pounce and kill by pumas in relation to deer mass. Points represent separate kills defined by pounce peak and immediate prey handling (Fig. 3 and table S3). The linear regression (black line, Eq. 4) for five known kills (black symbols) is extrapolated (red dashed line) to predict the size of an unidentified kill according to pounce energetics (red symbol).

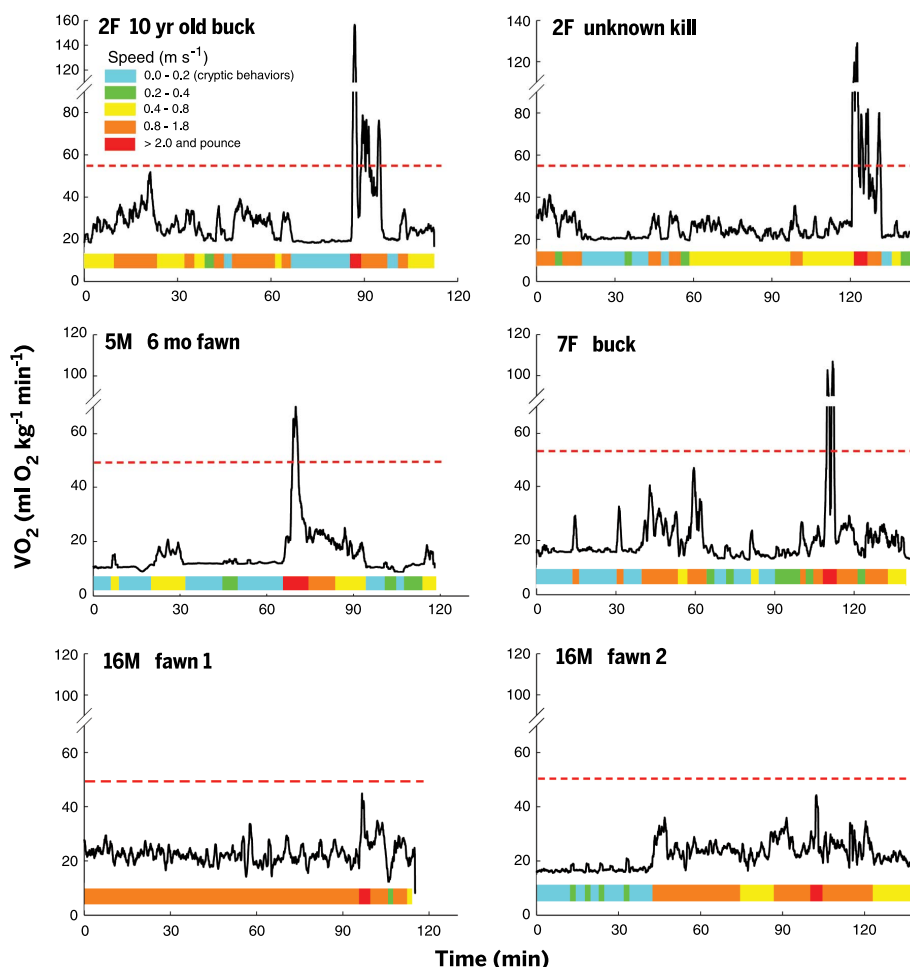


Fig. 3. Activity levels and energetic costs determined from ODBA of wild pumas. Each panel represents a separate kill identified by puma ID and by size and age of the deer. Two-hour hunt segments, including pre-kill, pounce spike, and post-kill prey handling periods, are compared for pumas 2F, 5M, 7F, and 16M. The red dashed line indicates predicted $\dot{V}O_{2\max}$ (20). Colored bars denote speed and behavior according to the inset scale. Low-cost periods (blue bars) were used to calculate percentage of time spent in cryptic activities for each puma.

(Fig. 2C). Extrapolating from this relationship, we estimated the size of an animal that had been attacked, despite being unable to view the event or carcass. Although some prey may be harder to kill and may require more handling than others, pumas appear to precisely gauge the magnitude of the initial pounce to account for the size of the animal to be overtaken (table S3).

We find that the investment of energy for traveling by large terrestrial carnivores is considerable and often underestimated. Comparing pre-kill transport costs for hunting pumas (COT_{HUNT} in $\text{J kg}^{-1} \text{m}^{-1}$) to those calculated for foxes (11, 12), wild dogs (10), and tigers (21) results in the allometric regression

$$COT_{\text{HUNT}} = 37.41(\text{mass}^{-0.30})$$

$$(r^2 = 0.74, n = 9 \text{ across 5 species}, P = 0.003)$$

(5)

(Fig. 4B) (13). Energetic demands for pre-kill movements by these large mammalian carnivores average 2.3 times the levels routinely used to model energetic costs from distances moved or GPS tracks (21–23) and 3.8 times the predicted COT_{MIN} (16, 24). These elevated costs undoubtedly reflect the metabolic demands of carnivory (16, 25) as well as the SMART collar's ability to account for previously overlooked energetic expenditures associated with intermittent locomotion, turning maneuvers, and kinematic changes due to uneven or variable substrates (26–28) that are often imperceptible in most GPS tracks (13). Pumas can mitigate high hunting costs by matching maximum pouncing force to prey size and using cryptic tactics. With such an energetic advantage, it is not surprising that felid morphology and physiology have been shaped over evolutionary time for the stalk-and-pounce sneak attack (5, 6).

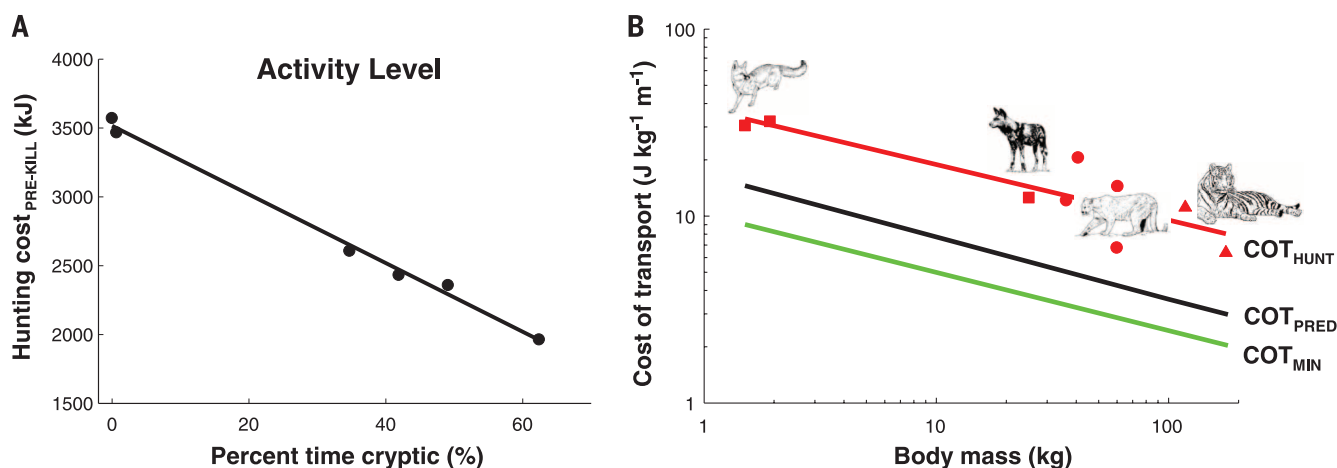


Fig. 4. Effects of behavior and body mass on energetic costs of hunting carnivores. (A) Total energy expended during the pre-kill periods in Fig. 3 in relation to percentage of time pumas spent performing low-activity level (cryptic) behaviors. See text for regression statistics. (B) Minimum (green line from Fig. 1), predicted [black line (14)], and measured (red line, Eq. 5) transport costs for hunting canids (squares) and felids (male and female pumas in this study, circles; male and female tigers, triangles). See supplementary materials for data sources.

Although our study highlights one energetically expensive activity (hunting) for a single carnivore, there are more than 245 mammalian carnivore species (www.iucnredlist.org) and numerous omnivores that pursue and kill mobile, elusive prey. Most have specialized hunting styles and would be expected to display highly variable instantaneous rates of energy expenditure that differ widely among predator and prey, habitats, and even individuals. The laboratory-to-field approach used here, which pairs basic physiological attributes of the animal with standard wildlife monitoring, provides a powerful way of quantifying such species-specific energetic demands. Some activities, including hunting and locomotion across complex habitats, are energetically costly; most are woefully underestimated by many predictive algorithms in common use. Correcting this will become increasingly important for the preservation of large carnivores as foraging becomes progressively affected by continued habitat degradation and loss of vegetative cover essential for cryptic movements (4, 29).

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Tables S1 to S3
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PROSTATE CANCER

Ubiquitylome analysis identifies dysregulation of effector substrates in SPOP-mutant prostate cancer

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Cancer genome characterization has revealed driver mutations in genes that govern ubiquitylation; however, the mechanisms by which these alterations promote tumorigenesis remain incompletely characterized. Here, we analyzed changes in the ubiquitin landscape induced by prostate cancer–associated mutations of SPOP, an E3 ubiquitin ligase substrate-binding protein. SPOP mutants impaired ubiquitylation of a subset of proteins in a dominant-negative fashion. Of these, DEK and TRIM24 emerged as effector substrates consistently up-regulated by SPOP mutants. We highlight DEK as a SPOP substrate that exhibited decreases in ubiquitylation and proteasomal degradation resulting from heteromeric complexes of wild-type and mutant SPOP protein. DEK stabilization promoted prostate epithelial cell invasion, which implicated DEK as an oncogenic effector. More generally, these results provide a framework to decipher tumorigenic mechanisms linked to dysregulated ubiquitylation.

Genome sequencing studies have revealed unanticipated roles for the ubiquitylation machinery in cancer. For example, the cullin-RING ubiquitin ligase adaptor protein speckle-type POZ protein (SPOP) is mutated in 8 to 14% of prostate and endometrial cancers (1–4). In prostate cancer, SPOP mutations are confined to specific amino acid residues within the substrate-binding cleft, which mediates substrate interaction and ubiquitin transfer (5). This exquisite localization suggests that SPOP mutations have undergone positive selection during tumorigenesis by altering binding and ubiquitylation of distinct effector substrates in a protumorigenic manner. However, the mecha-

nisms and substrates underlying this phenomenon remain incompletely understood.

In principle, mutant SPOP could enhance ubiquitylation of SPOP substrates (gain-of-function effect) or ubiquitylate new substrates (neomorphic effect). Alternatively, SPOP mutants could dimerize with their wild-type counterparts (e.g., through the BTB and BACK domains), and so repress wild-type SPOP function (dominant-negative effect). In support of the latter model, SPOP mutations typically occur in a heterozygous state with a retained wild-type allele and are able to dysregulate known substrates [e.g., nuclear receptor coactivator 3 (NCOA3)] in a dominant-negative manner (1, 6).

Characterizing the ubiquitin landscape (or “ubiquitylome”) that results from cancer genomic alterations affecting ubiquitin ligase components may provide new insights into tumorigenesis. To interrogate changes in ubiquitylation conferred by prostate cancer SPOP mutations, we stably overexpressed vector control (C); wild-type SPOP (SPOP-WT); or a mutated variant (SPOP-F133L or SPOP-Y87N, in which Phe¹³³ is replaced by Leu or Tyr⁸⁷ is replaced by Asn (SPOP-MT)), in immortalized prostate epithelial cells expressing endogenous SPOP (fig. S1, A and B) (7). In each case, we characterized the resulting ubiquitylome by measuring glycine-glycine remnants of ubiquitylated lysines (K-ε-GG) after

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trypsin digestion and stable isotope labeling of amino acids in cell culture (SILAC)-based mass spectrometry (Fig. 1A). To account for ubiquitylation-related changes in protein expression, all K- ϵ -GG values were normalized to protein abundance (Fig. 1A) (database D1 in supplementary materials).

We reasoned that putative K- ϵ -GG peptide substrates might show distinct patterns of protein abundance depending on whether mutant SPOP caused a dominant-negative, loss-of-function, gain-of-function, or neomorphic effect. In a dominant-negative context, substrate peptides should exhibit a [SPOP-WT > control > SPOP-MT] pattern of abundance, whereas a pure loss-of-function effect might yield a [SPOP-WT > control = SPOP-MT] pattern. In contrast, a gain-of-function effect may result in a [SPOP-MT > SPOP-WT > control] pattern, and a neomorphic effect should cause a [SPOP-MT > SPOP-WT = control] pattern.

Only 12 of 7181 K- ϵ -GG peptides detected exhibited more than twofold changes in peptide abundance in the SPOP-mutant contexts (z score >3 or <-3) compared with control or SPOP-WT (Fig. 1B; fig. S1, C and D; table S1; and database

D1). Differentially regulated (more than twofold) K- ϵ -GG peptides generally followed a dominant-negative pattern, including those corresponding to WIZ, SCAF1, GLYR1, DEK, and TRIM24 proteins (Fig. 1B and figs. S1, C and D, and S2, A to E). K- ϵ -GG peptides from two proteins (corresponding to G3BP1 and CAPRIN1) showed a loss-of-function pattern (figs. S1, C and D, and S2, F and G), and two K- ϵ -GG peptides from SPOP itself showed up-regulation in the setting of both SPOP-WT and SPOP-MT overexpression, suggestive of autoubiquitylation (fig. S1, C and D). Although we observed no evidence for gain-of-function or neomorphic effects, we cannot exclude the existence of such mechanisms.

We next determined whether SPOP-binding motifs were enriched in the differentially regulated proteins. Indeed, of the seven proteins described above (excluding SPOP itself), three (43%) contained a previously characterized SPOP-binding sequence (DEK, SCAF1, and CAPRIN1) (5). In contrast, the frequency of SPOP-binding sites was significantly lower (~3%) across the ubiquitylome as a whole ($P < 0.001$) (table S2). We identified one

K- ϵ -GG peptide corresponding to DAXX, a known SPOP substrate (8). As expected, this peptide was enriched—and the corresponding total protein was down-regulated—after overexpression of wild-type SPOP (fig. S3A). Expression of the transcriptional coactivator NCOA3, another SPOP substrate, followed a dominant-negative pattern, as reported previously (6, 9) (fig. S3B).

Because protein ubiquitylation is often a prerequisite for proteasomal degradation, we next sought to ascertain which differentially expressed K- ϵ -GG peptides showed an inverse correlation with total protein expression (database D1). Peptides corresponding to TRIM24, SCAF1, and GLYR1 fit this pattern (one peptide each) (Fig. 1C and fig. S3, C and D). TRIM24 has been shown to mediate ligand-dependent activation of the androgen receptor—a key prostate cancer driver—and to degrade TP53 through its E3 ligase activity (10, 11). This observation is of interest given that primary prostate cancers with SPOP mutations typically lack TP53 alterations (1).

However, the K- ϵ -GG peptides that exhibited the most profound down-regulation coupled with

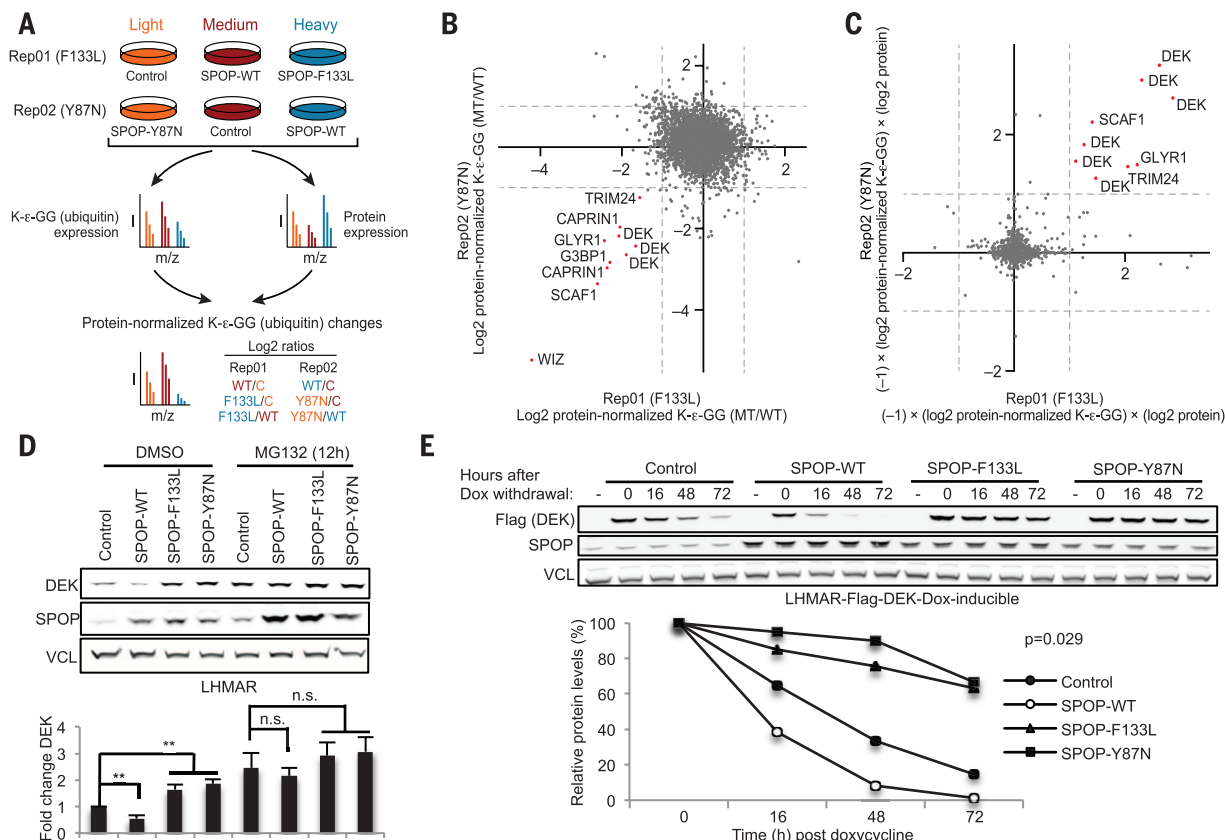


Fig. 1. K- ϵ -GG (ubiquitin)-profiling and validation for DEK in immortalized prostate epithelial cells (LHMAR) overexpressing vector control, SPOP-WT and SPOP-F133L/Y87N mutants (MT). (A) Schematic showing the design of the proteomics experiments. Isogenic cell line expressing either vector control, SPOP-WT or SPOP-MT were isotopically labeled (see fig. S1, A and B). K- ϵ -GG ratios were normalized to protein expression changes, assessed in parallel. (B) Scatter plot of protein normalized K- ϵ -GG level changes between SPOP-MT and SPOP-WT (depicted as log2 ratio of MT/WT) shows coordinate down-regulation of distinct K- ϵ -GG peptides across replicates (see also figs. S1, C and D, and S2, A to G). (C) To visualize K- ϵ -GG sites

that undergo inverse changes at the protein level (e.g., more than twofold), normalized K- ϵ -GG data shown in (B) was inverted and multiplied by corresponding total protein expression changes (see also fig. S3, C and D, for expression changes). (D) DEK protein expression across isogenic LHMAR cells assessed by immunoblotting with and without proteasome inhibition by MG132. DMSO, dimethyl sulfoxide. Error bars represent means $\pm$ SD, $n = 4$ (n.s., not significant; ** $P < 0.01$, Student's t test). (E) Degradation of Flag-DEK over time. Flag-DEK was induced by doxycycline (Dox) and protein decay measured by immunoblotting after doxycycline withdrawal in inducible LHMAR cells (P value, Friedman test). See also fig. S3E for DEK mRNA expression.

robust (e.g., more than twofold) up-regulation of the corresponding total protein mapped to DEK (Fig. 1C). DEK is a putative oncoprotein, first described as a fusion gene in an aggressive subset of acute myeloid leukemia (12–14). This finding therefore raised the possibility that DEK might represent an SPOP substrate that becomes dysregulated by oncogenic prostate cancer SPOP mutations.

We next determined whether prostate cancer SPOP mutants affected DEK protein stability. Indeed, whereas forced expression of SPOP-WT in immortalized prostate epithelial cells reduced DEK protein levels, the SPOP-F133L and SPOP-Y87N mutants produced an accumulation of DEK (Fig. 1D). Treatment with the proteasome inhibitor MG132 also increased DEK, consistent with a possible role for the proteasome in DEK regulation.

We also examined the effect of SPOP mutants on DEK protein stability, using immortalized prostate epithelial cells engineered to express a doxycycline-inducible Flag-tagged DEK. After doxycycline exposure, Flag-DEK decayed more rapidly in control cells than in cells containing SPOP mutants (Fig. 1E); there was no difference in DEK mRNA expression (fig. S3E). These results support the notion that prostate cancer SPOP mutants function in a dominant-negative fashion to impair ubiquitylation and proteasomal degradation of DEK.

Given that forced expression of SPOP reduced DEK protein levels, we sought to determine whether SPOP interacts with DEK directly to promote ubiquitylation. In support of this hypothesis, the primary DEK amino acid sequence contained a five-amino acid consensus SPOP-binding motif

(fig. S3F) (5). To test this, we first overexpressed a Flag-tagged DEK protein harboring serine-to-alanine mutations at the motif (S287A/S288A) and assessed the ability of wild-type SPOP to repress DEK expression. As predicted, the S287A/S288A variant abolished the repressive effect of SPOP and produced elevated levels of DEK protein (Fig. 2A). To determine whether this motif mediated direct binding of SPOP to DEK, we performed immunoprecipitation experiments in cells expressing either wild-type DEK or the S287A/S288A variant. Whereas overexpressed SPOP protein was detectable after immunoprecipitation of Flag-DEK (Fig. 2B) and at the endogenous level (fig. S4A), the DEK(S287A/S288A) variant disrupted the DEK-SPOP interaction (Fig. 2B). Thus, the SPOP-binding motif within DEK appeared necessary for SPOP binding and destabilization.

Next, we tested whether SPOP could ubiquitylate DEK as part of a larger CUL3-RBX1 complex (8). In vitro, the addition of CUL3 and RBX1 caused wild-type DEK to migrate as high-molecular-weight species indicative of a multiple monoubiquitylation pattern (Fig. 2C and fig. S4B). In contrast, DEK (S287A/288A) failed to undergo efficient ubiquitylation (Fig. 2C). Addition of an ubiquitin moiety that is unable to form polyubiquitin chains because of lysine-to-arginine substitutions produced a similar pattern (KO) (fig. S4C), which supported the idea that SPOP may promote “multimonoubiquitylation” of DEK. This result aligns well with the observation that multiple ubiquitylated DEK lysine residues were detected in the initial proteome analysis (fig. S2E).

To determine whether DEK ubiquitylation targets this protein for proteasomal degradation, we cultured SPOP- and DEK-expressing cells in the presence or absence of the proteasome inhibitor MG132. Short-term (4- to 5-hour) MG132 treatment markedly increased ubiquitylated Flag-DEK, with a smaller effect on total protein expression (Fig. 2D and fig. S4D). Prolonged proteasomal inhibition (16 hours), or depletion of SPOP with two independent lentiviral short hairpin RNAs (shRNAs), also increased DEK protein levels (fig. S4E). Moreover, SPOP knockdown increased DEK stabilization after doxycycline was removed by using the inducible system described above, without affecting DEK mRNA levels (fig. S4, F and G). In aggregate, these data are consistent with a model in which multimonoubiquitylation of DEK promotes its proteasomal degradation, in line with recent studies showing that multiple monoubiquitylation can be sufficient for proteasomal targeting (15). The lower affinity of monoubiquitin moieties (as opposed to ubiquitin chains) for the proteasome may explain why DEK protein turnover is relatively slow (Fig. 1, D and E, and fig. S4F) (15).

We next asked whether prostate cancer SPOP mutations might disrupt the interaction between SPOP and DEK. Neither SPOP-F133L nor SPOP-Y87N could ubiquitylate DEK effectively in vitro (Fig. 2E). Moreover, both of these SPOP mutants suppressed ubiquitylation induced by SPOP-WT (Fig. 2E) and decreased basal DEK ubiquitylation in cell culture (fig. S5A). In prostate epithelial cells,

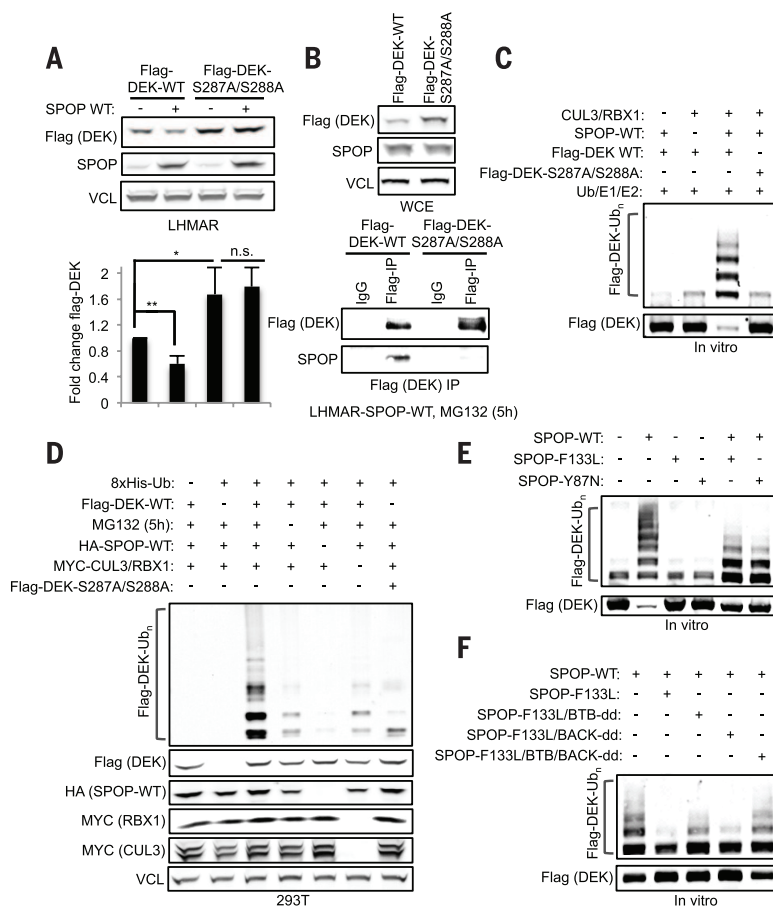


Fig. 2. DEK is a SPOP substrate and SPOP mutants repress DEK ubiquitylation. (A) Effects of stable transduced wild-type SPOP overexpression on protein levels of Flag-DEK and corresponding S287A/S288A mutant were assessed by immunoblotting in LHMAR cells. Error bars represent means $\pm$ SD, $n = 4$ (n.s., not significant; $*P < 0.05$, $**P < 0.01$, Student's t test). (B) Flag-immunoprecipitation of Flag-DEK and corresponding S287A/S288A mutant in LHMAR cells with forced SPOP expression. (C) In vitro ubiquitylation of DEK. Flag-DEK was purified by Flag-specific antibody immunoprecipitation from LHMAR cells and incubated with the indicated components. Flag-DEK immunoprecipitates were analyzed by immunoblotting (see also fig. S4B). (D) In vivo ubiquitylation of DEK. Human embryonic kidney 293T cells were transfected with ubiquitin (Ub) tagged with eight histidines (8xHis-ubiquitin) and indicated constructs, with or without MG132, and lysed. Immunoblot of protein lysates and 8xHis-ubiquitin pull down using nickel beads. (E) In vitro ubiquitylation as in (C) using indicated recombinant SPOP components in equimolar ratios. See also fig. S5A. (F) In vitro ubiquitylation as in (E) using indicated recombinant SPOP components. Dimerization-deficient mutants of SPOP-F133L: BTB domain (L186D/L190D/L193D/I217K = BTB-dd) and BACK domain (Y353E = BACK-dd). See also fig. S6.

overexpression of mutant SPOP resulted in increased total SPOP and DEK protein expression, as expected. However, both SPOP-F133L and SPOP-Y87N substantially reduced the interaction of wild-type SPOP with Flag-DEK in immunoprecipitation assays (fig. S5B). These data thus support the notion that prostate cancer SPOP mutants may impair DEK ubiquitylation in a dominant-negative manner.

Suppression of ubiquitylation by mutant SPOP may conceivably occur through heteromeric (e.g., WT-mutant) SPOP complexes. Along these lines, SPOP is known to undergo dimerization and multimerization through its BTB and BACK domains (5, 16, 17). In support of this model, Flag-tagged

SPOP-F133L coimmunoprecipitated with hemagglutinin (HA)-tagged SPOP-WT (fig. S6A). To determine whether dimerization might mediate the suppressive function of SPOP-F133L, we introduced dimerization-deficient mutations involving the BTB (L186D/L190D/L193D/I217K; BTB-dd) or BACK (Y353E; BACK-dd) domains into SPOP-F133L (5, 17). Both dimerization-deficient mutants abolished formation of high-molecular-weight SPOP complexes and retained CUL3 binding, as evidenced by size-exclusion chromatography (fig. S6B). Similarly, HA-tagged versions of these SPOP mutants showed appropriate subcellular localization, and coimmunoprecipitated with MYC-tagged CUL3 (fig. S6, C and D). However, both the BTB

and BACK domain mutations reversed the suppressive effect of SPOP-F133L mutant on DEK ubiquitylation in vitro (Fig. 2F) and in vivo (fig. S6E). In particular, the dimerization-deficient mutations impaired the interaction of wild-type SPOP with Flag-DEK, on the basis of immunoprecipitation experiments (fig. S6F). In aggregate, these data support the notion that prostate cancer SPOP mutations exert a dominant-negative effect on wild-type SPOP activity through formation of heteromeric complexes.

Next, we assessed whether DEK might contribute to oncogenic phenotypes induced by mutant SPOP in prostate cancer. Toward this end, both DEK and SPOP have previously been implicated in cell invasion (1, 18). We found that SPOP-F133L and SPOP-Y87N induced collagen invasion by prostate epithelial cells, whereas wild-type SPOP suppressed invasion (Fig. 3A). Similar effects were observed in the human model of prostate cancer (LNCaP) cells (fig. S7A). To assess the contribution of DEK to this phenotype, we overexpressed DEK in cells expressing SPOP-WT or an empty vector control, and analyzed the resulting invasion phenotypes. In both settings, DEK overexpression promoted cellular invasion (Fig. 3B). Similarly, overexpression of Flag-DEK-S287A/S288A—the mutant form of DEK that cannot bind SPOP—also increased invasion, which implies that this phenotype is not simply a consequence of trapped SPOP-DEK complexes (fig. S7B). Conversely, shRNA knockdown of DEK decreased cellular invasion in cells overexpressing SPOP-Y87N (Fig. 3B) but the effect on control cells was minimal (fig. S7C). These results suggest that DEK may influence the invasive phenotype conferred by prostate cancer SPOP mutations, although a SPOP-independent role for DEK in cellular invasion cannot be ruled out.

DEK has also been implicated in the maintenance of stem cell-like properties, such as sphere formation under nonadherent culture conditions (14, 18–20). Consistent with these observations, SPOP-Y87N produced elevated DEK levels and enhanced sphere formation in primary human

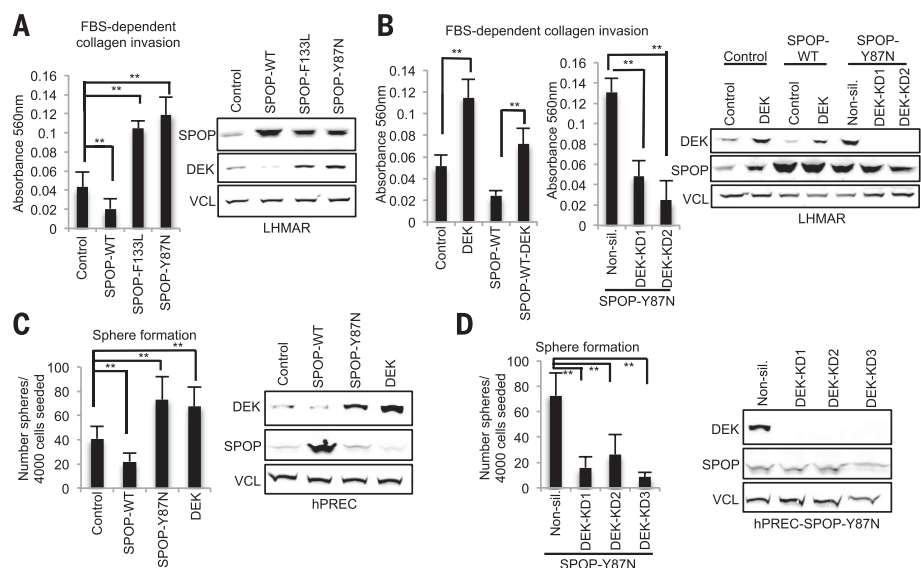
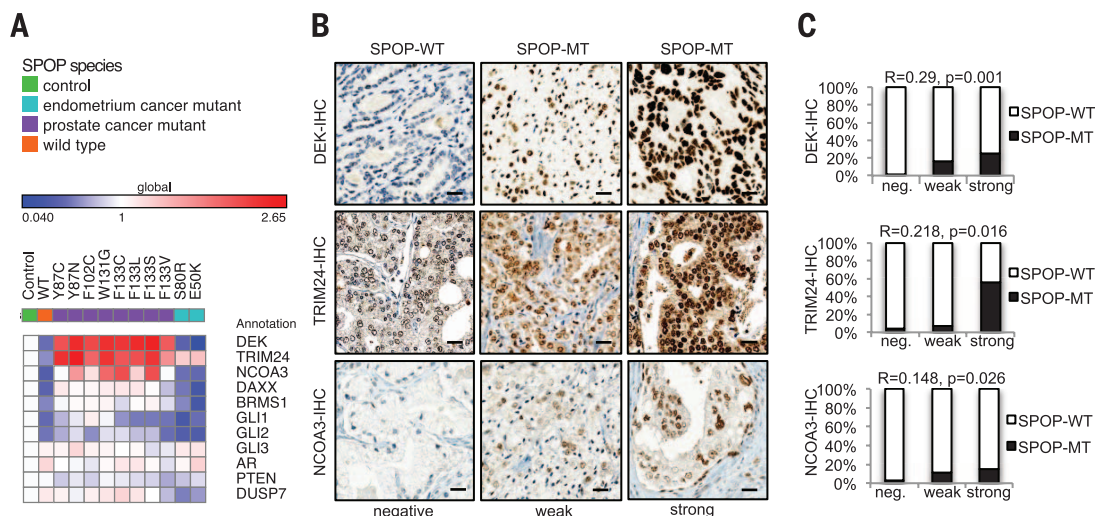


Fig. 3. SPOP mutant-related changes in DEK expression contribute to invasion and sphere formation. (A) Effects of different SPOP species on collagen invasion and DEK expression in LHMAR cells. FBS, fetal bovine serum. (B) Collagen invasion changes related to forced expression and depletion of DEK with two shRNAs in LHMAR cells. See also fig. S7. (C). Effects of different SPOP species and DEK on sphere formation in primary prostate epithelial cells (hPREC). (D) Effects of DEK depletion by three shRNAs on sphere formation. Error bars represent means $\pm$ SD, $n = 3$ in triplicate (n.s., not significant; $**P < 0.01$, Student's t test). See also fig. S7D.

Fig. 4. Up-regulation of DEK, TRIM24, and NCOA3 is a feature of prostate cancer SPOP mutations.

(A) Effects of different SPOP species on abundance of indicated proteins in unmodified primary prostate epithelial cells (hPREC) by immunoblotting. Protein changes relative to individual controls depicted as median in a heat map ($n = 3$). (B) Representative images of primary prostate cancer tissues stained for DEK, TRIM24, and NCOA3 by immunohistochemistry. Scale bar, 30 μ m. (C) Corresponding expression analysis on 181/178 primary tumors stratified according to their SPOP mutations status (P values, Kendall beta-tau). See also figs. S8 to S10.



prostate epithelial cells (hPREC) (Fig. 3C and fig. S7D). In contrast, overexpression of SPOP-WT suppressed sphere formation in this setting (Fig. 3C and fig. S7D). Overexpression of DEK in hPREC cells also augmented sphere formation, whereas shRNA depletion of DEK (with three independent shRNAs) in cells overexpressing SPOP-87N impaired sphere-forming capacity (Fig. 3D and fig. S7D). Taken together, these data suggest that SPOP-dependent DEK regulation may promote a stemlike phenotype.

We then investigated whether the effects of SPOP mutations on DEK regulation might be generalizable across a broad spectrum of cancer-associated SPOP mutations. Here, we tested SPOP mutants from both prostate and endometrial cancer for their effects on DEK protein levels, collagen invasion, and sphere formation. All prostate cancer-associated SPOP mutants examined [representing >80% of mutations identified in this tumor type (1)] conferred increased DEK protein expression, whereas two recurrent endometrial cancer-associated SPOP mutants (S80R and E50K) (2, 3) had no effect (Fig. 4A and figs. S8A, S9A, and S10A) in prostate cells. As expected, DEK expression correlated with sphere-forming capacity and cell invasion without affecting baseline cell proliferation or viability (figs. S8, B and C, and S9B).

Finally, we considered whether other putative SPOP effector proteins—both known substrates and novel ones nominated by our ubiquitylome screen—might also undergo dysregulated expression in the setting of prostate cancer SPOP mutations (8, 9, 21–23). Regarding the latter, we noted that TRIM24 represented another putative SPOP substrate from our screen with potential biological relevance to prostate cancer (Fig. 1, B and C, and fig. S3C) (10, 11). As expected several known or putative SPOP substrates were down-regulated in response to SPOP-WT overexpression (including DEK, TRIM24, NCOA3, DAXX, BRMS1, and GLI1 and 2) (Fig. 4A and fig. S10A). However, only DEK, TRIM24, and NCOA3 also became up-regulated upon overexpression of prostate cancer SPOP mutants in this setting (6). Moreover, nuclear expression of these proteins correlated significantly with SPOP mutations in 181/178 primary prostate cancers analyzed by immunohistochemistry (Fig. 4, B and C, and fig. S10B, table S3). These data are consistent with the ubiquitylome and experimental studies, which supports a model in which SPOP mutations may engage overlapping routes of transformation in prostate cancer by dysregulation of DEK, TRIM24, and NCOA3.

These data support a model wherein prostate cancer SPOP mutants impair ubiquitylation and degradation of a subset of SPOP substrates in a dominant-negative manner to promote tumorigenesis. Future studies of DEK dysregulation and its biological functions in normal and tumor tissue may therefore promote new biological insights relevant to many human cancers. More generally, large-scale profiling of the ubiquitin landscape linked to tumor genomic alterations in protein homeostasis genes may catalyze the identification of downstream effector mechanisms. Given the growing number of such genes

identified by comprehensive cancer genome studies, this approach may provide an additional avenue for functional annotation of the cancer genome. In the future, these studies could uncover new biological and therapeutic avenues that exploit dysregulated protein homeostasis or ubiquitin-based regulation in cancer.

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SUPPLEMENTARY MATERIALS

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AGING

Aging-induced type I interferon response at the choroid plexus negatively affects brain function

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Aging-associated cognitive decline is affected by factors produced inside and outside the brain. By using multiorgan genome-wide analysis of aged mice, we found that the choroid plexus, an interface between the brain and the circulation, shows a type I interferon (IFN-I)-dependent gene expression profile that was also found in aged human brains. In aged mice, this response was induced by brain-derived signals, present in the cerebrospinal fluid. Blocking IFN-I signaling within the aged brain partially restored cognitive function and hippocampal neurogenesis and reestablished IFN-II-dependent choroid plexus activity, which is lost in aging. Our data identify a chronic aging-induced IFN-I signature, often associated with antiviral response, at the brain's choroid plexus and demonstrate its negative influence on brain function, thereby suggesting a target for ameliorating cognitive decline in aging.

One of the mysteries of brain senescence is to what extent it is influenced by aging of other body tissues (1, 2). Recent studies have suggested that age-related changes in both circulating soluble factors (3–5) and peripheral immunity (6) are involved in this process. Nevertheless, given the fact that the mammalian central nervous system (CNS) is secluded behind barriers from directly interacting with the blood circulation (7), the underlying mechanism remains unclear.

The choroid plexus (CP), an epithelial monolayer that forms the blood–cerebrospinal fluid (CSF) barrier and produces the CSF (8), serves as a neuroimmunological interface in shaping brain function in health and pathology by integrating signals from the brain with signals coming from the circulation (6, 9–11). We envisioned that understanding how CP activity is altered in aging might lead to identification of strategies to attenuate aging-associated cognitive decline.

To determine whether aging of the CP reflects aging of the brain and other body tissues, we systematically characterized the mRNA expression profile of young (3-month-old) and aged (22-month-old) murine organs by RNA sequencing. Whereas all examined tissues from aged mice showed increased expression of mRNA transcripts associated with age-related processes, such as immune response ($P = 2.66 \times 10^{-05}$; cluster XIX; fig. S1, A and B), the aged CP exhibited an expression profile corresponding to type I interferon (IFN-I) response ($P = 4.25 \times 10^{-07}$; cluster IX; Fig. 1A and fig. S1, A to C), classically associated with antiviral activity (12). To exclude an aging-unrelated effect such as viral infection, we confirmed the increased expression of IFN-I-dependent genes [e.g., IFN regulatory factor 7 (*irf7*), IFN- β 1 (*ifn β*), and IFN-induced protein with tetratricopeptide repeats 1 (*ifit1*)] by real-time quantitative polymerase chain reaction (qPCR) in different cohorts of aged mice from various animal centers

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(Fig. 1B). Closer examination by qPCR and immunohistochemical staining showed that, whereas IFN-I-associated gene expression was increased in the aged CP, expression of type II IFN (IFN- γ)-dependent genes (10) [e.g., intercellular adhesion molecule 1 (*icam1*), IFN- γ -induced protein 10 (*cxcl10*), and chemokine (C-C motif) ligand 17 (*ccl17*)] was decreased (Fig. 1, C and D). Immunohistochemical staining of brain sections of mice, as well as postmortem brain sections from non-CNS-diseased humans, confirmed an aging-associated increase of IFN-I at the CP (Fig. 1E) and suggested that this signature is conserved among species.

The CP epithelium is exposed to brain-derived signals from its apical side, via the CSF, and to peripheral signals from its basal side, via the circulation (8). To differentially examine which of these compartments induces the transcriptional signature of the aged CP, we used a heterochronic parabiosis model (13), in which young and aged mice were surgically connected to share vasculature. Under these conditions, there are bidirectional, although not symmetric, effects on hippocampal neurogenesis and cognitive function between young and aged heterochronic parabionts (3, 4). Transcriptome analyses revealed that the circulation of aged mice failed to induce expression of IFN-I-dependent genes in the CP of young heterochronic partners. It did, however,

affect expression of IFN-II-dependent genes, including reduced expression of homing and trafficking determinants required for physiological leukocyte entry via the CP to the CSF [Fig. 2, A and B, and fig. S2, A and B (clusters III and VIII)] (10) and increased expression of *ccl11* (Fig. 2C), a chemokine that impairs brain plasticity (3), and its expression by the CP is induced by the cytokine interleukin (IL)-4 when local IFN- γ levels are diminished (6). Under the same experimental setting, the young circulation failed to reverse the expression program of IFN-I-dependent genes in the aged CP yet affected expression of IFN-II-dependent genes (10) (Fig. 2, A and B, and fig. S2, A and B). However, CCL11 expression by the CP of the aged heterochronic parabionts was not affected by the young circulation (Fig. 2C), suggesting that IFN-II regulation of CCL11 expression in the aged CP involves additional factors that are not derived from the circulation. These results indicated that the IFN-II expression program at the aged CP is modulated by both circulation- and brain-derived factors, whereas IFN-I in this compartment might be induced by factors in the CSF emitted from the aged brain. Therefore, we exposed primary cultures of CP epithelial cells of young mice to CSF aspirated from aged or young mice; we found that the CSF of aged but not of young mice induced the IFN-I-dependent response (Fig. 2D).

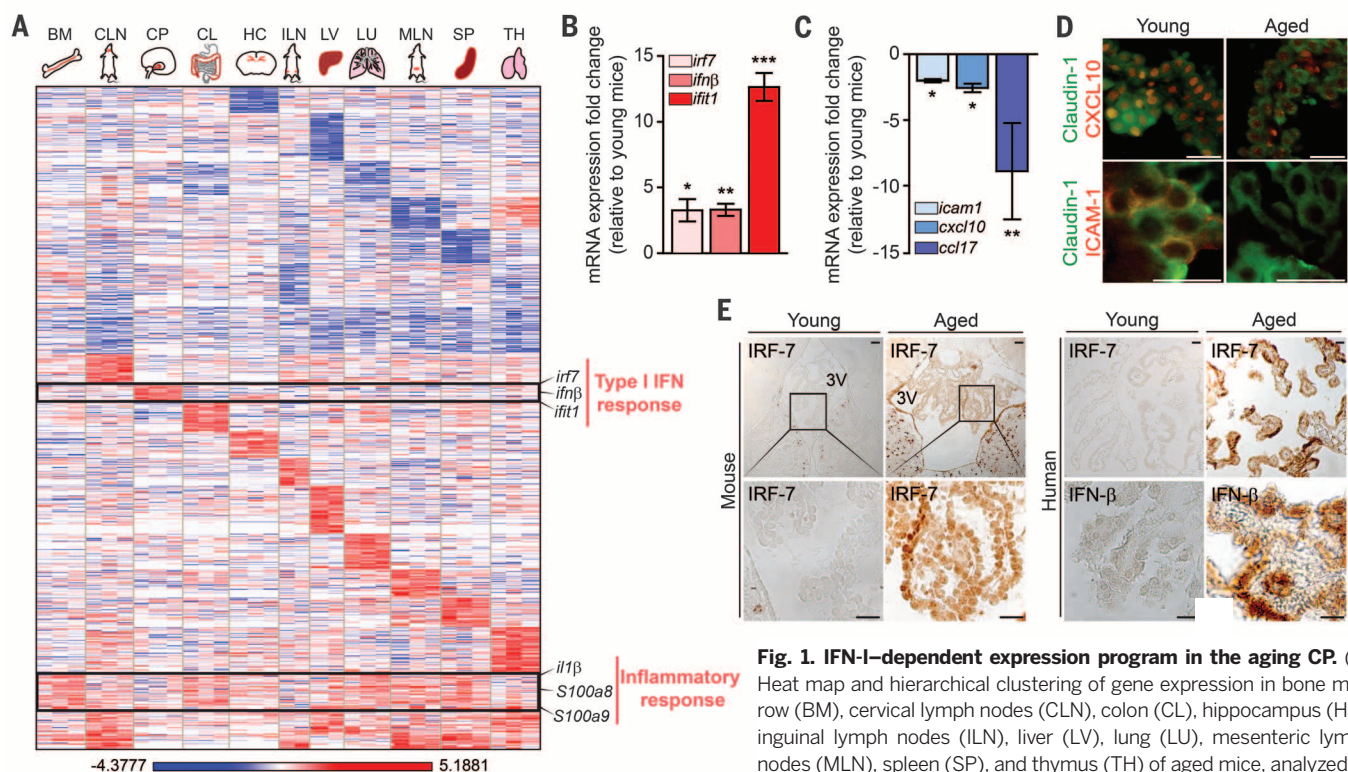


Fig. 1. IFN-I-dependent expression program in the aging CP. (A)

Heat map and hierarchical clustering of gene expression in bone marrow (BM), cervical lymph nodes (CLN), colon (CL), hippocampus (HC), inguinal lymph nodes (ILN), liver (LV), lung (LU), mesenteric lymph nodes (MLN), spleen (SP), and thymus (TH) of aged mice, analyzed by RNA sequencing. Results are displayed as log of expression relative to

young controls ($n = 2$ or 3). (B and C) mRNA abundance of IFN-I- and IFN-II-dependent genes in the CP of aged animals (fold change relative to young mice CPs; $n = 10$ mice per group; bars represent mean $\pm$ SEM; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; Student's t test for each pair; data are representative of at least three independent experiments). (D) Representative images of brain sections of young and aged mice, immunostained for claudin-1 (epithelial tight junctions; in green) and either CXCL10 or ICAM1 (in red) (scale bars indicate 25 μ m). (E) Representative micrographs of immunohistochemical staining for IRF7 in young and aged mice and for IRF7 and IFN- β in young and aged human postmortem CPs (scale bars, 50 μ m; 3V, third ventricle).

This response was recapitulated when epithelial cells of the CP were treated with a mixture of proinflammatory cytokines that accumulate in the brain during aging and neurodegeneration (14) (fig. S3).

The apparent inverse relations between type I and type II IFN expression programs in the aged CP (Figs. 1 and 2) prompted us to examine their relevance to CP and brain function. Followup of transgenic mice deficient in IFN-II signaling, including IFN- γ receptor knockout (*IFN- γ R*^{-/-}) mice and *Tbx21*^{-/-} mice [impaired in IFN- γ production by CD4⁺ T cells (15)], revealed that these animals developed spatial learning and memory deficits (fig. S4, A to H) and displayed reduced hippocampal neurogenesis during

adulthood (fig. S4, I to K). Further examination of *IFN- γ R*^{-/-} mice showed that brain functional decline was accompanied by impaired CP activity in supporting physiological leukocyte trafficking to the CSF (fig. S4, L and M). Thus, impaired ability to respond to IFN- γ or reduced IFN- γ in the circulation resulted in cognitive decline and restricted hippocampal neurogenesis during adulthood, before chronological aging (fig. S5). Our observations, together with the essential role of IFN-II in CP function (10) and findings outside the CNS that chronically activated IFN-I signaling interferes with IFN-II-dependent resolution of inflammation (16–18), led us to examine whether chronic IFN-I in the aged CP could affect brain function and, if so,

whether it involves interfering with local IFN-II-dependent activities.

In vitro exposure of CP epithelial cells to the major IFN-I cytokine, IFN- β , induced a classical “antiviral” response (Fig. 3A) and resulted in reduced expression of insulin-like growth factor (*igf1*) and brain-derived neurotrophic factor (*bdnf*) (Fig. 3B), molecules that support neuronal growth, differentiation, and survival and are produced by the CP (11). In vivo administration of neutralizing antibodies to the IFN-I receptor (α -IFNAR) to the CSF of aged mice blocked the IFN-I response program at the CP (Fig. 3C) and resulted in restoration of *igf1* and *bdnf* expression (Fig. 3D and fig. S6), as well as *ccl11* expression (Fig. 3E), to levels similar to those found in the CP of young

Fig. 2. Effect of signals from the CSF but not from the blood circulation on IFN-I-dependent genes in the CP. (A) Heat map and hierarchical clustering of gene expression in the CPs of aged (A) and young (Y) iso- (A-A or Y-Y) and hetero- (A-Y) chronic parabionts, analyzed by RNA sequencing and displayed as log of expression relative to young, isochronic CPs ($n = 2$ or 3). (B and C) mRNA expression of IFN-I-dependent genes, *ifit1* and *irf7* (B), and the IFN-II-dependent gene, *ccl11* (C), in the CPs of parabiotic mice ($n = 6$ to 8 mice per group). (D) mRNA expression of *ifit1* and *irf7* in primary cultures of CP cells treated with phosphate-buffered saline (PBS) or CSF of young or aged mice ($n = 4$ or 5 per group). Throughout the figure, bars represent mean $\pm$ SEM; N.S., not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; one-way analysis of variance (ANOVA) with Newmann-Kleus post hoc test.

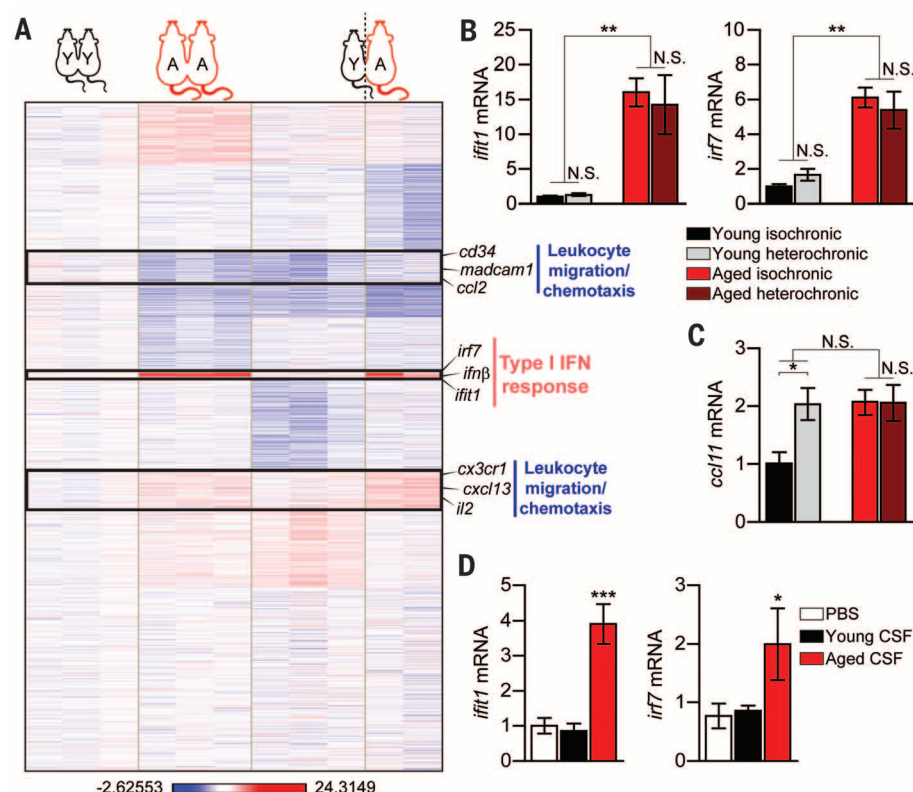
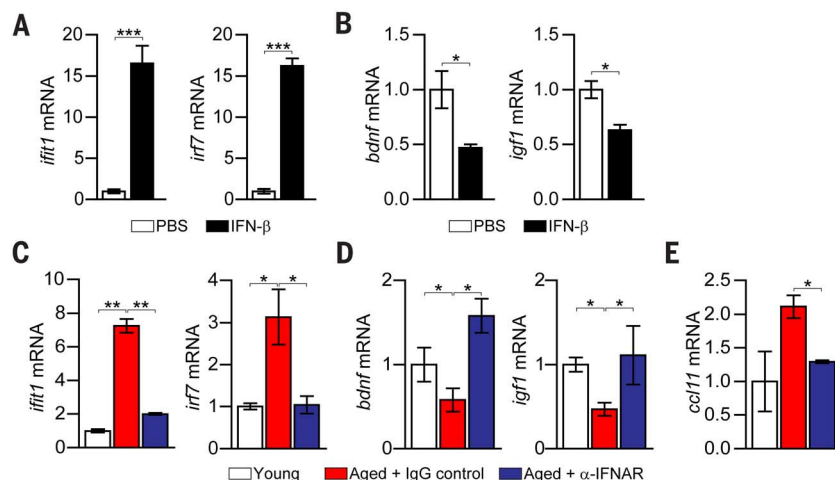


Fig. 3. Restored CP function after neutralization of the aging-induced type I IFN response in the brain. (A and B) *ifit1* and *irf7* (A) and *bdnf* and *igf1* (B) mRNA expression in CP epithelial cells cultured for 24 hours with murine IFN- β ($n = 4$ per group; bars represent mean $\pm$ SEM; * $P < 0.05$; *** $P < 0.001$, Student's *t* test). (C) *ifit1* and *irf7* mRNA expression in the CP 3 days after α -IFNAR or IgG intracerebroventricular (i.c.v.) administration. (D and E) *bdnf* and *igf1* (D) and *ccl11* (E) mRNA expression in the CP 7 days after α -IFNAR or IgG control i.c.v. administration ($n = 6$ or 7 per group). Bars represent mean $\pm$ SEM; * $P < 0.05$; one-way ANOVA with Newmann-Kleus post hoc test.



mice. Thus, neutralizing the IFN-I response within the brain affected CP function and suggested that chronically elevated IFN-I interferes with IFN-II activities in the aged CP.

To determine whether *in vivo* neutralization of IFN-I in aging could reverse cognitive dysfunction, we assessed two different measurements of brain plasticity, hippocampal-dependent spatial memory and hippocampal neurogenesis (19) (Fig. 4A). Because age-associated cognitive decline varies across individuals, we first scored memory ability in aged mice by the novel location recognition (NLR) task, in which animals explore two objects, and the exploration time of each object is measured. After 24 hours, one of the objects is relocated, and mice with normal memory spend more time exploring the displaced object, compared with the object that was not moved (20). Cognitively impaired aged mice selected this way (Fig. 4, A and B, and fig. S7) received either α -IFNAR or immunoglobulin G (IgG) injected into their CSF and were tested again in new settings of an NLR test. α -IFNAR-

treated mice, unlike the control group, showed improved memory (Fig. 4C), suggesting that neutralizing IFN-I signaling within the brain could restore cognitive function in aged mice. Moreover, the number of newborn neurons, stained for 5-bromo-2'-deoxyuridine (BrdU) (21) and doublecortin (DCX), was increased in the hippocampus of the α -IFNAR-treated group (Fig. 4D), reaching levels comparable to those of aged mice that maintained cognitive function; enhanced hippocampal neurogenesis to a similar extent in aged mice was correlated with improved cognitive function (4). Examination of the hippocampal parenchyma for the effect on age-related neuroinflammation (2), which detrimentally affects adult neurogenesis (22, 23), revealed increased mRNA expression of the anti-inflammatory cytokine, IL-10 (Fig. 4E), and a marked decrease in astrogliosis and microgliosis (Fig. 4, F and G) in the α -IFNAR-treated group.

Although originally identified by their antiviral properties, type I IFNs have important roles in attenuating inflammation, both outside and

inside the CNS (24–26). Nevertheless, we found that blocking IFN-I signaling in the brain of aged mice partially restored cognitive function and hippocampal neurogenesis and attenuated age-related chronic neuroinflammation. It is thus possible that the IFN-I response program at the aged CP represents a physiological mechanism evoked to mitigate age-related neuroinflammation. When this process is not timely terminated, it becomes detrimental to brain plasticity, at least in part by interfering with IFN-II activity in the CP and brain parenchyma, which is needed for physiological immune-dependent brain maintenance and resolution of neuroinflammation in aging and pathology (6, 9, 10, 27–29) (fig. S8). Other blood-brain interfaces may undergo similar changes in aging. Our findings may shed light on the association between cerebral levels of IFN-I and memory impairments in several human diseases (25) and the neurological and neuropsychiatric complications that often accompany IFN-I administration to patients (30). Therefore, neutralizing the

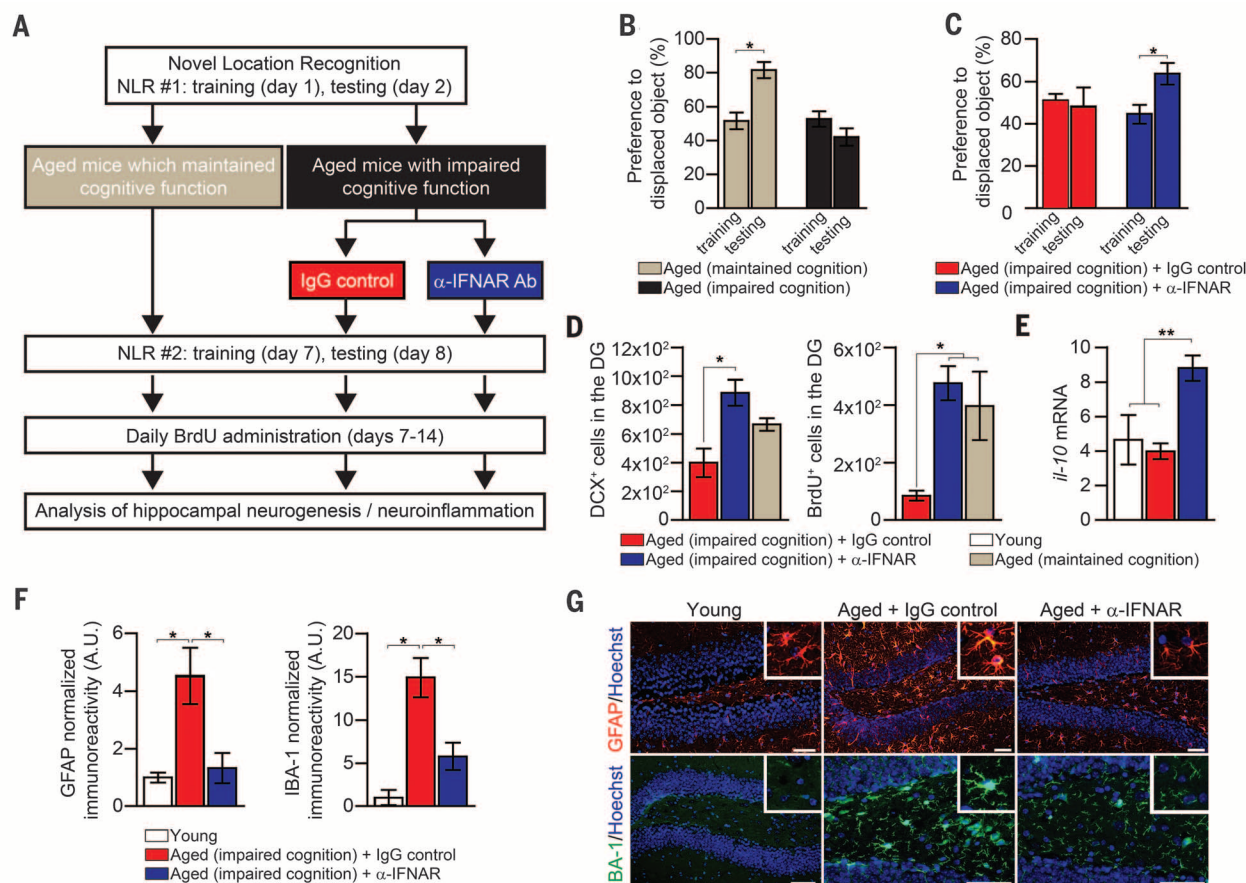


Fig. 4. Restored brain function after neutralization of the age-induced type I IFN response in the brain. (A) Scheme illustrating the experimental design. (B) Performance of aged mice in the first NLR task ($n = 5$ to 18 per group). (C) Performance of α -IFNAR- and IgG-injected cognitively impaired aged mice in the second NLR task ($n = 5$ or 6 per group). (D and E) Analysis of the hippocampal dentate gyrus (DG) of α -IFNAR or IgG intracerebroventricularly injected cognitively impaired aged mice 14 days after treatment and of young and aged mice that maintained cognitive function ($n = 4$ to 6 per

group). Quantification of hippocampal DCX- and BrdU-labeled newborn neurons (young mice DCX- and BrdU-labeled cell counts were $16,832 \pm 873$ and 4403 ± 51 , respectively) (D); *il-10* mRNA expression in the hippocampus (E). (F and G) Quantification (F) and representative images (G) of glial fibrillary acidic protein (GFAP) and ionizing calcium binding adaptor molecule 1 (IBA-1) staining in hippocampal sections (Hoechst in blue; scale bars, 50 μ m). Throughout the figure, bars represent mean $\pm$ SEM; * $P < 0.05$; ** $P < 0.01$; one-way ANOVA with Newmann-Kleus post hoc test.

response to type I IFNs within the CNS might provide an approach to reverse or slow down age-associated cognitive decline or other pathological conditions associated with chronic IFN-I within the CNS.

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Materials and Methods

Figs. S1 to S8

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T CELL MEMORY

A local macrophage chemokine network sustains protective tissue-resident memory CD4 T cells

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CD8 tissue-resident memory T (T_{RM}) cells provide efficient local control of viral infection, but the role of CD4 T_{RM} is less clear. Here, by using parabiotic mice, we show that a preexisting pool of CD4 T_{RM} cells in the genital mucosa was required for full protection from a lethal herpes simplex virus 2 (HSV-2) infection. Chemokines secreted by a local network of macrophages maintained vaginal CD4 T_{RM} in memory lymphocyte clusters (MLCs), independently of circulating memory T cells. CD4 T_{RM} cells within the MLCs were enriched in clones that expanded in response to HSV-2. Our results highlight the need for vaccine strategies that enable establishment of T_{RM} cells for protection from a sexually transmitted virus and provide insights as to how such a pool might be established.

Effector memory T cells circulate throughout the peripheral tissues, and although they are sufficient to provide protection against systemic infections (1–4), they cannot control infection localized to peripheral tissues (4–6). After viral skin infection, CD8 tissue-resident memory T (T_{RM}) cells control infection more efficiently than circulating CD8 T cells (7–10) and recruit circulating T cells to the sites of infection (11). In contrast to CD8 T_{RM} cells, little is known regarding the existence or importance of CD4 T_{RM} cells. Unlike CD8 T cells, memory CD4 T cells readily traffic through circulation to provide protection at distal sites (12). Respiratory infection with influenza virus induces protective CD4 T cells capable of migrating and establish-

ing residency in the lung (13). However, to what extent resident versus circulating memory CD4 T cells are required for protection of the host and the mechanism by which such a memory pool is maintained are unclear.

To address these questions, we used a mouse model of genital herpes. Intravaginal (ivag) immunization with an attenuated herpes simplex virus 2 (HSV-2) strain [thymidine kinase-negative (TK⁻ HSV-2)] results in complete protection from secondary challenge with wild-type (WT) HSV-2 (14). TK⁻ HSV-2 replicates in the vaginal keratinocytes and establishes latent infection in the dorsal root ganglia but does not reactivate (15). The protective memory response to HSV-2 vaginal challenge requires CD4 T cells that secrete the cytokine interferon- γ (IFN- γ) but does not require either CD8 T cells or B cells (16). Although all routes of immunization induced systemic CD4 T cell responses, only ivag immunization with TK⁻ HSV-2 recruited significant numbers of viral-specific

CD4 T cells to the vagina (17, 18) and conferred protection against lethal disease (fig. S1), which indicated that the local immunization is required to mount protective immunity.

To examine the selective ability of local immunization to confer protective immunity, we used parabiotic mice. Full chimerism was achieved in systemic circulation within 2 weeks of parabiosis (fig. S2) (19). The vaginal memory pool, analyzed 6 weeks after parabiosis of ivag TK⁻ HSV-2 immune pairs, was largely disconnected from the rest of the circulation (Fig. 1A). Host-derived CD4 T_{RM} cells were mainly distributed within memory lymphocyte clusters (MLCs), whereas the rare donor-derived circulating CD4 T cells were found at the periphery of the MLCs (Fig. 1B). In contrast, CD8 T_{RM} cells resided within the epithelium and within the MLCs, and circulating CD8 T cells were rarely found (Fig. 1B). Within the MLCs, CD11c⁺ and major histocompatibility complex (MHC) class II⁺ cells were present (fig. S3, A and B). Unlike the tertiary lymphoid organs that contain peripheral node addressin-positive (PNAd⁺) high endothelial venules and a developed lymphatic endothelial network (20), PNAd⁺ blood vessels and lymphatic vessel endothelial hyaluronin acid receptor 1-positive (LYVE-1⁺) lymphatic endothelial vessels were not found within the MLCs (fig. S3). Other features typical of tertiary lymphoid tissues were also absent, such as germinal center B cells (fig. S3D) and naïve lymphocytes (fig. S4C).

HSV-2-specific CD4⁺ T_{RM} cells were T cell receptor $\alpha\beta$ -positive (TCR $\alpha\beta$ ⁺) (fig. S4A) and secreted IFN- γ , tumor necrosis factor- α (TNF- α), and interleukin 2 (IL-2) (fig. S4B) in response to viral antigen stimulation. The cell-surface phenotype of CD4⁺ T_{RM} cells resembled that of CD8 T_{RM} cells (CD62L^{hi} CD44^{hi} CD11a⁺ CD69⁺ CD49d⁺ CCR7) (7, 21) (fig. S4, B and C). Expression of KLF2, a zinc-finger transcription factor whose suppression promotes retention of CD8 T_{RM} cells in tissues (22)—and its downstream genes *S1pr1* and *Sell* (CD62L)—were also suppressed in CD4

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T_{RM} cells residing within the MLCs (Fig. 1C). Only a small subset of $CD4^+ T_{RM}$ cells expressed CD103 (fig. S4, B and C), a molecule important for $CD8^+ T_{RM}$ cell survival within the epithelia (23).

Next, to investigate whether a continuous supply of circulating lymphocytes is required to maintain the vaginal memory pool, mice immunized intravaginally were treated with FTY720,

an antagonist of sphingosine-1-phosphate receptor 1 (S1PR1) that blocks lymphocyte egress from lymph nodes. Although circulating lymphocytes were depleted from peripheral blood

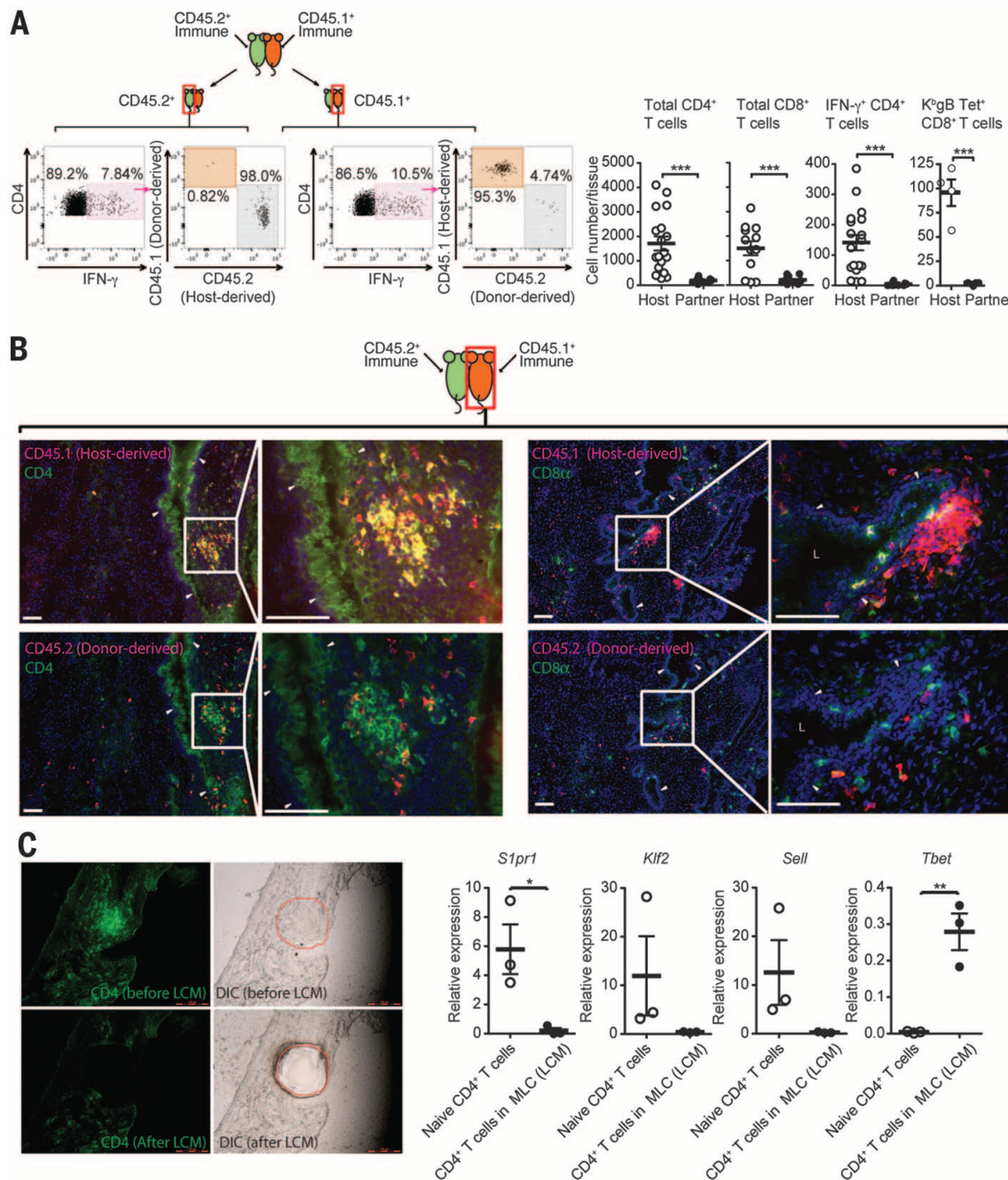


Fig. 1. Vagina-resident memory T cells are found in MLCs. $CD45.2^+$ and $CD45.1^+$ mice immunized with TK $^-$ HSV-2 5 weeks before were surgically joined. **(A)** Six weeks later, the presence of total and HSV-specific host and donor $CD4^+$ and $CD8^+$ T cells in vaginal tissues of parabiotic mice was analyzed by flow cytometry. Host-derived and partner-derived cell numbers of total $CD4^+$ and $CD8^+$ T cells ($n = 6$ to 9 pairs), HSV-2-specific $IFN-\gamma^+$ $CD4^+$ T cells ($n = 9$ pairs), and K^b gB tetramer $^+$ $CD8^+$ T cells ($n = 2$ pairs) in vaginal tissues of parabiotic mice were analyzed by flow cytometry. These data were combined from multiple experiments and analyzed by two-tailed unpaired t test. **(B)** Three weeks after parabiosis, frozen sections of vaginal tissue were stained with antibodies against $CD4$ or $CD8$ (green) and $CD45.1$ or $CD45.2$ (red). The images were captured by using a 10 $\times$ or

40 $\times$ objective lens. Arrows indicate the basement membrane. Scale bars indicate 100 μ m. These are representative of two pairs of parabiosed mice. **(C)** Frozen section of vaginal tissues of C67BL/6 mice immunized with TK $^-$ HSV-2 (5 weeks) was stained with antibody against $CD4$. $CD4^+$ cells in MLCs were collected by laser capture microdissection (LCM). LCM samples were pooled from three vaginal tissues, four or five slides per mouse. The images were captured using a 20 $\times$ objective lens. Scale bars indicate 100 μ m. DIC, differential interference contrast. qRT-PCR analysis of indicated mRNA was performed in $CD4^+$ T cells in MLCs and naive $CD4^+$ T cells that were sorted from spleen ($n = 3$). Their gene expression was normalized to that of *Cd4* for each sample. Data are means $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (two-tailed unpaired t test).

and accumulated in the lymph nodes after 2 weeks of FTY720 treatment (fig. S5, A and B) (24), the number of HSV-specific IFN- γ ⁺ CD4 T cells and K^b glycoprotein B (gB) tetramer⁺ CD8 T cells (fig. S5) in the vagina remained unchanged. Thus, MLCs, the main site for CD4 T_{RM} cell residency, are distinct from tertiary lymphoid organs and do not require circulating cells for maintenance.

Next, we compared the ability of tissue-resident versus circulating memory lymphocytes to mediate protection against a lethal HSV-2 challenge using various parabiotic combinations. Mice relying on only the circulating memory cells (group 4) were impaired in their ability to suppress viral replication, and about half of this group succumbed to viral disease, in contrast to the fully protected mice in group 3 that harbor T_{RM} cells (Fig. 2, A to C, and fig. S6). Transient virus control seen in group 4 (first 2 days of challenge) is likely mediated by HSV-2-specific immunoglobulin G (IgG), as previously reported (25, 26). Protection conferred by the circulating memory cells depended on CD4⁺ T cells of the immune donor mice (group 5) but not CD8⁺ T cells (group 6). In addition, IFN- γ responsiveness by the cells within the challenged animal was required for protection by WT circulating memory T cells (group 7) (Fig. 2, A to C, and fig. S6). Thus, circulating CD4 memory T cells are able to provide some level of protection against HSV-2 infection and disease, by secreting IFN- γ and inhibiting viral replication in the vaginal epithelium, an observation consistent with skin HSV-1 infection (12). However, for complete protection from disease and mortality, local CD4 T_{RM} cells are crucial

in providing antiviral defense within the genital mucosa.

Why are circulating memory CD4 T cells inferior to T_{RM} cells? After vaginal infection of the naïve partner with WT HSV-2, circulating T cells from the immunized partner entered the tissue within 2 days (Fig. 2D, filled bars). However, the newly arriving circulating HSV-2-specific memory CD4 T cells did not secrete IFN- γ until 5 days after challenge with HSV-2 (Fig. 2E) but not an irrelevant virus, influenza (figs. S7 and S8). Because rapid delivery of IFN- γ to the vaginal epithelium is crucial in HSV-2 control (27), this may be why circulating HSV-specific memory CD4 T cells provided poor protection. In contrast, in the presence of MLCs, circulating HSV-2-specific memory T cells were barely recruited upon ivag secondary challenge with viruses (fig. S8). These data indicated that the alarming function of T_{RM} cells (17) might be trumped by the abundance and efficiency of preexisting T_{RM} cells within the MLCs in clearing the secondary infection.

To examine the antigen-specificity of T_{RM} cells, we analyzed the sequence of the CDR3 region of TCR β gene of CD4 T cells isolated from MLCs by laser capture microdissection. Six TCR β CDR3 sequences were shared by CD4⁺ T cells in MLCs and those in the draining lymph nodes (DLNs) at the peak of primary immune response in TK⁻ HSV-2 immunized mice, but not in naïve mice (Fig. 3A). One of these sequences, CTCSAAGGDAEQFF (TCRV β 01-01*01 and TCRJ β 02-01*01) [with variable (V) and joining (J) regions as described], represented a dominant clone (33%) in the CD4 T cells in the MLCs (Fig. 3, B and C). This

dominant clone was also detected in the DLNs and in a portion (11%) of CD4 T cells residing outside of MLCs (Fig. 3, B and C). Furthermore, we found that HSV-2-specific CD4 T_{RM} cells expressed TCRV β 01-01*01 (Fig. 3D and fig. S9). These results indicated that MLCs are highly enriched for HSV-2-specific CD4 T_{RM} cells.

We next addressed the mechanism by which T_{RM} cells are maintained in the vagina. Treatment of TK⁻ HSV-2 intravaginally immunized mice with pertussis toxin (PTX), which inhibits G α_i signaling downstream of many chemokine receptors, resulted in a considerable reduction in HSV-specific memory T cell numbers in the vagina and CD4 T_{RM} cells expressing TCR V β CDR3 sequence (CTCSAAGGDAEQFF) in MLCs (figs. S9B and S10A), the disappearance of the MLCs (fig. S10B), and lack of protection against ivag HSV-2 challenge (fig. S10C). The disappearance of T_{RM} cells was not associated with cell death (fig. S11A) but was due to the expulsion of CD4 T cells into the vaginal lumen (fig. S11, B and C). These data suggested that chemokine receptor signaling is required for the retention of T_{RM} cells within the MLCs. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) revealed selective up-regulation of CCL5 and CXCL9 in vaginal tissue at 5 and 12 weeks after TK⁻ HSV-2 immunization, and this expression was largely dependent on IFN- γ (Fig. 4A). Expression of CCR1, CCR5, and CXCR3 was detected in the CD4 T_{RM} and CD8 T_{RM} cells by flow cytometry (fig. S12A). To determine whether these chemokines are required for the retention of T_{RM} cells within the vagina, we treated HSV-2-immunized mice with

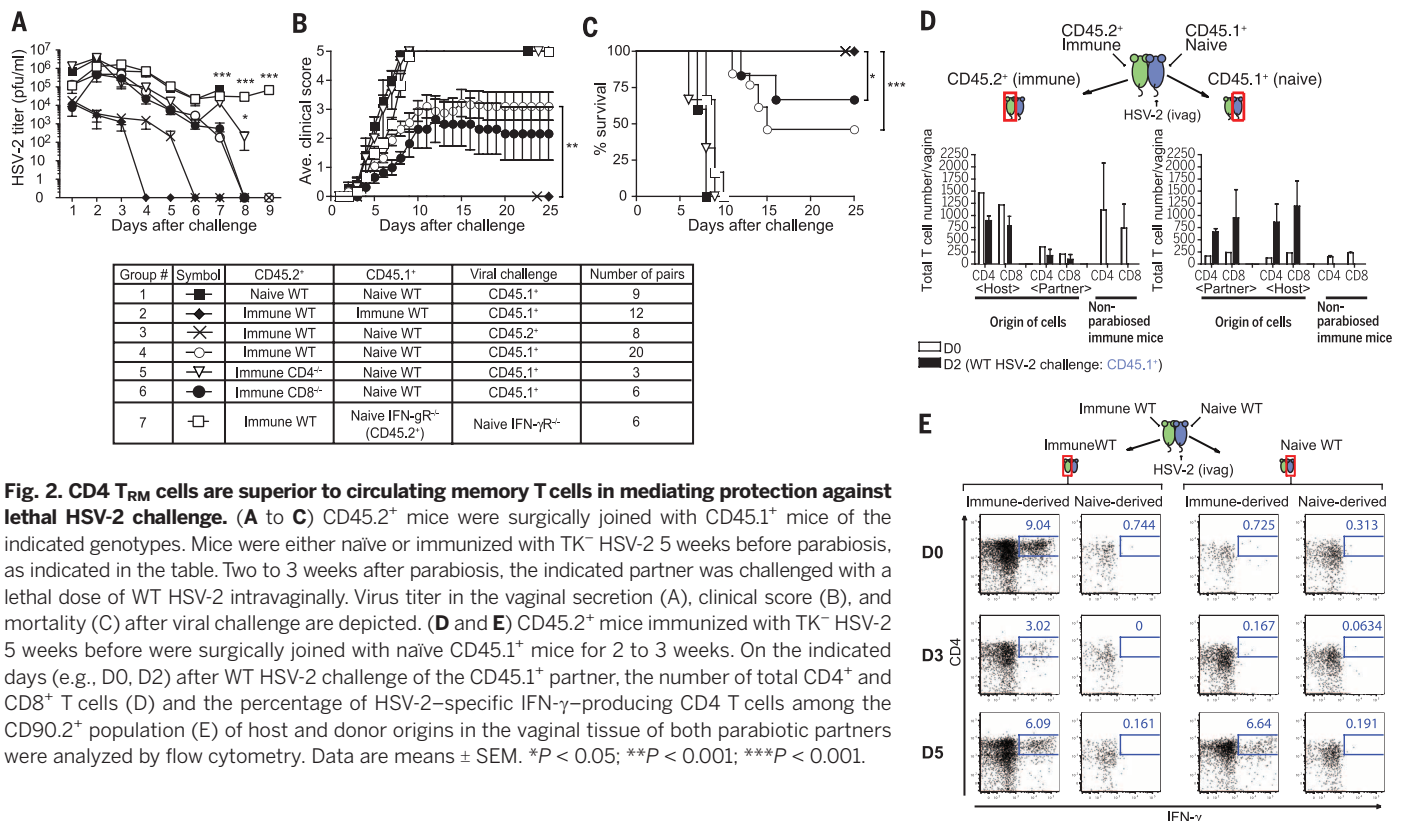


Fig. 2. CD4 T_{RM} cells are superior to circulating memory T cells in mediating protection against lethal HSV-2 challenge. (A to C) CD45.2⁺ mice were surgically joined with CD45.1⁺ mice of the indicated genotypes. Mice were either naïve or immunized with TK⁻ HSV-2 5 weeks before parabiosis, as indicated in the table. Two to 3 weeks after parabiosis, the indicated partner was challenged with a lethal dose of WT HSV-2 intravaginally. Virus titer in the vaginal secretion (A), clinical score (B), and mortality (C) after viral challenge are depicted. (D and E) CD45.2⁺ mice immunized with TK⁻ HSV-2 5 weeks before were surgically joined with naïve CD45.1⁺ mice for 2 to 3 weeks. On the indicated days (e.g., D0, D2) after WT HSV-2 challenge of the CD45.1⁺ partner, the number of total CD4⁺ and CD8⁺ T cells (D) and the percentage of HSV-2-specific IFN- γ -producing CD4 T cells among the CD90.2⁺ population (E) of host and donor origins in the vaginal tissue of both parabiotic partners were analyzed by flow cytometry. Data are means $\pm$ SEM. * P < 0.05; ** P < 0.001; *** P < 0.001.

neutralizing antibodies against CXCL9 and CCL5. The dose of antibody used was deemed saturating (fig. S13). Blockade of CCL5 diminished the number of HSV-2-specific CD4⁺ T_{RM} and CD8 T_{RM} cells in the vagina (Fig. 4, B and C, and fig. S12B) and led to a partial collapse of MLCs (fig. S14A). CXCL9 blockade reduced the number of CD8 T_{RM} cells. It is noteworthy that neutralizing CCL5 resulted in diminished protective immune re-

sponses to secondary challenge with HSV-2 ivag infection to the level similar to CD4⁺ T cell depletion (Fig. 4C). Thus, these data indicate that CD4⁺ T_{RM} cells are maintained through continuous interaction with the chemokine CCL5 and that this interaction is required for protection from secondary challenge.

Next, we determined the localization and cellular source of CCL5 within the MLC. Five weeks

after ivag immunization, CCL5⁺ cells were mainly localized within MLCs and were maintained for at least 13 weeks (fig. S14B). Furthermore, the CCL5 producers within the MLCs were hematopoietic (CD45⁺), and were CD11b⁺ CD11c^{lo} (Fig. 4D). By flow cytometry, CCL5 expression was detected in mainly macrophages (Autofluorescence^{hi}, CD11c^{lo}, CD11b⁺, MerTK⁺, CD64⁺) (28), with variable expression of MHC II (fig. S15) but not T cells

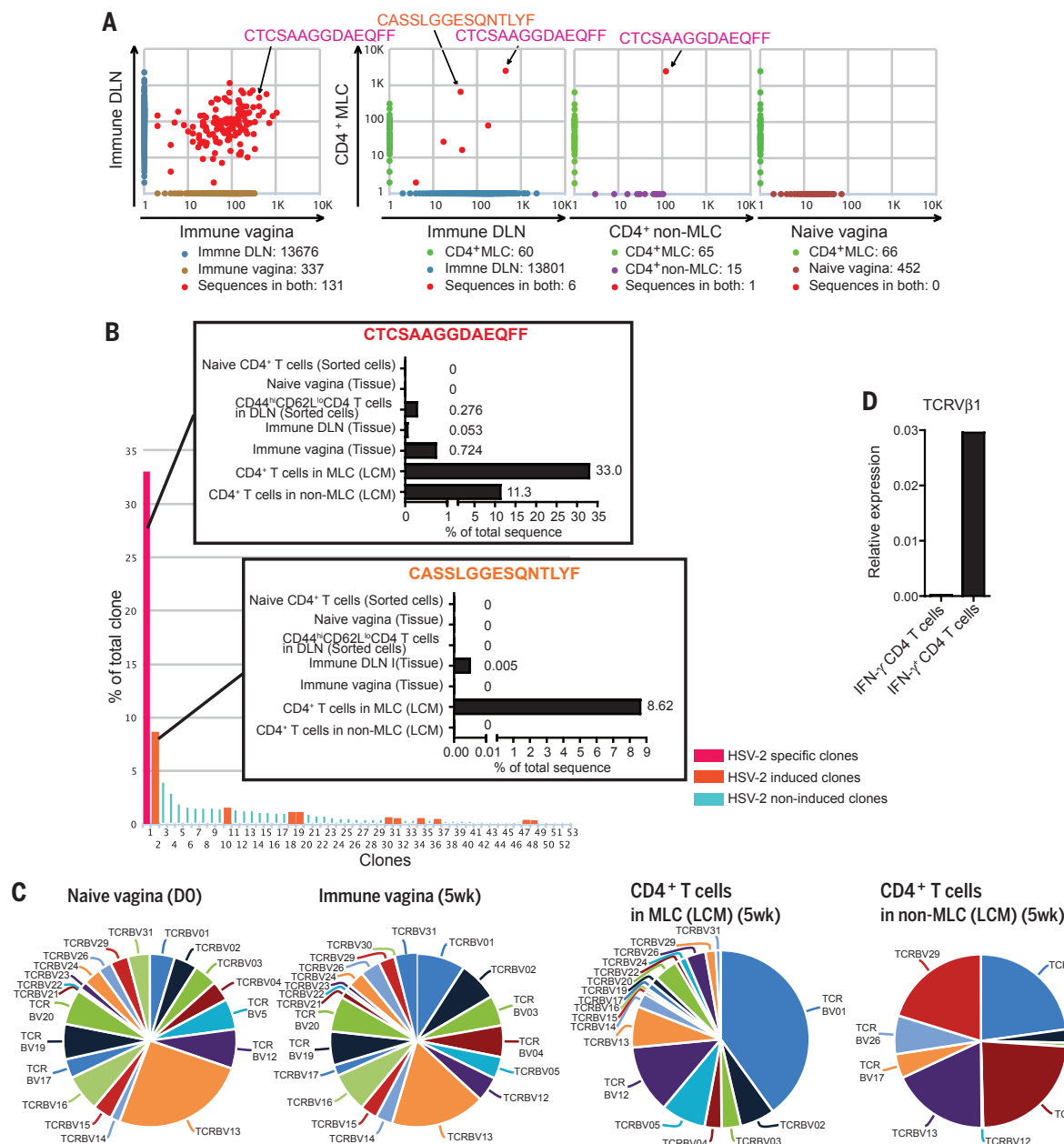


Fig. 3. Analysis of CD4⁺ TCR β-chain repertoires in MLCs. Frozen sections of vaginal tissues of C57BL/6 mice immunized with TK⁻ HSV-2 (for 5 weeks) were stained with antibody against CD4. CD4⁺ cells within the MLCs and non-MLC tissue were collected by LCM (pooled from vaginal tissues from 10 mice). DNA from LCM samples, total vaginal tissues of immunized mice, sorted naive CD44^{lo}CD62L^{hi} CD4⁺ T cells, and sorted CD44^{hi}CD62L^{lo} CD4⁺ T cells in DLNs 7 days after TK⁻ HSV-2 immunization (pooled from at least three mice) were subjected to TCR β-chain sequencing. (A) Common CDR3 sequences of TCR β chains between the indicated samples are depicted. (B) In MLCs, 53 clones

of TCR β-chain sequences were detected. Percentage of total clones for HSV-2-specific clones (pink), HSV-2-induced clones (orange), and HSV-2 noninduced clones (blue) are depicted. (C) The pie charts show the frequency of TCR Vβ families in vaginal tissue (naïve and immune), CD4⁺ T cells in MLCs, and non-MLC tissue. (D) Single-cell suspensions from vaginal tissues of mice vaginally immunized with TK⁻ HSV-2 5 weeks before were restimulated with splenocytes loaded with HSV-2 antigen. IFN-γ⁺ CD4⁺ and IFN-γ⁻ CD4⁺ cells as shown in fig. S9C were sorted for qPCR analysis of TCR Vβ1. Gene expression of these was normalized to that of *GAPDH* for each sample.

(fig. S15, C and F), and the number of CCL5⁺ cells increased after immunization (fig. S15E). Staining for CCL5 was specific (fig. S15, B and D). Depletion of CD11b⁺ cells in diphtheria toxin receptor-inducible CD11bDTR→WT bone marrow chimera led to a reduction of CCL5⁺ cells in the vagina (fig. S16, C and D), complete elimination of MLC structure, and diminished CD4⁺ T_{RM} cells in the vaginal tissue within 5 days (Fig. 4E and fig. S16, A and B). Collectively, our results suggest a model in which CD4⁺ T_{RM} cells are maintained through a positive-feedback loop, in which CD4⁺ T_{RM} cell stimulation by local macrophages results in low IFN- γ secretion, which in turn induces chemokines, such as CCL5, to retain the T cells within the MLCs. Although there was no detectable viral genomic DNA or mRNA expression in the vagina (fig. S17), it remains possible that small amount of viral peptide presentation by MHC II is required for the feedback loop. In support of this hypothesis, single cells were isolated from the vagina in the absence of ex vivo stimulation, and

secreted IFN- γ was captured onto the cell surface and stained. This uncovered two types of cell populations—those that constitutively express intermediate levels of IFN- γ and those that secrete high levels of IFN- γ after HSV-2 secondary challenge but not after an irrelevant virus infection (influenza) (fig. S18). The majority of the IFN- γ ^{int} cells (where superscript int indicates intermediate) were CD4⁺ T_{RM} cells (fig. S19, A and B) and required the presence of CD11b⁺ cells for their IFN- γ production (fig. S16F). In contrast, after WT HSV-2 secondary challenge, antigen-presenting cells that had captured HSV-2 antigen induced the secretion of large amounts of IFN- γ by CD4⁺ T_{RM} cells in an antigen-dependent manner (figs. S18 and S19C). Therefore, these results support a model in which CD4⁺ T_{RM} cells are maintained by chemokines secreted from local macrophages, which are in turn induced by basal levels of IFN- γ continuously secreted by T_{RM} cells (fig. S20). Upon infection with HSV-2, the CD4⁺ T_{RM} cells are stimulated by cognate

antigen to secrete high levels of IFN- γ , which results in viral clearance.

Our study highlights the importance of local memory T cells in antiviral defense against genital HSV-2 infection and supports the hypothesis that a local T cell presence correlates with viral control on the basis of human studies (29–31). Circulating memory T cells failed to access the genital mucosa at steady state. After infection, whereas circulating memory CD4⁺ T cells do enter the genital tissue and provide protection, viral control is both delayed and incomplete. In contrast, preexisting CD4⁺ T_{RM} cells in the MLCs were able to respond rapidly to secondary challenge and suppressed replication before the virus spread to the nervous system. MLCs are maintained through interaction between CD4⁺ T_{RM} cells and macrophages, which results in T cell secretion of IFN- γ and induction and expression of CCL5 by the local macrophages. Similar MLC-like structures have been reported in humans infected with HSV-2 (32) and *Chlamydia trachomatis* (33),

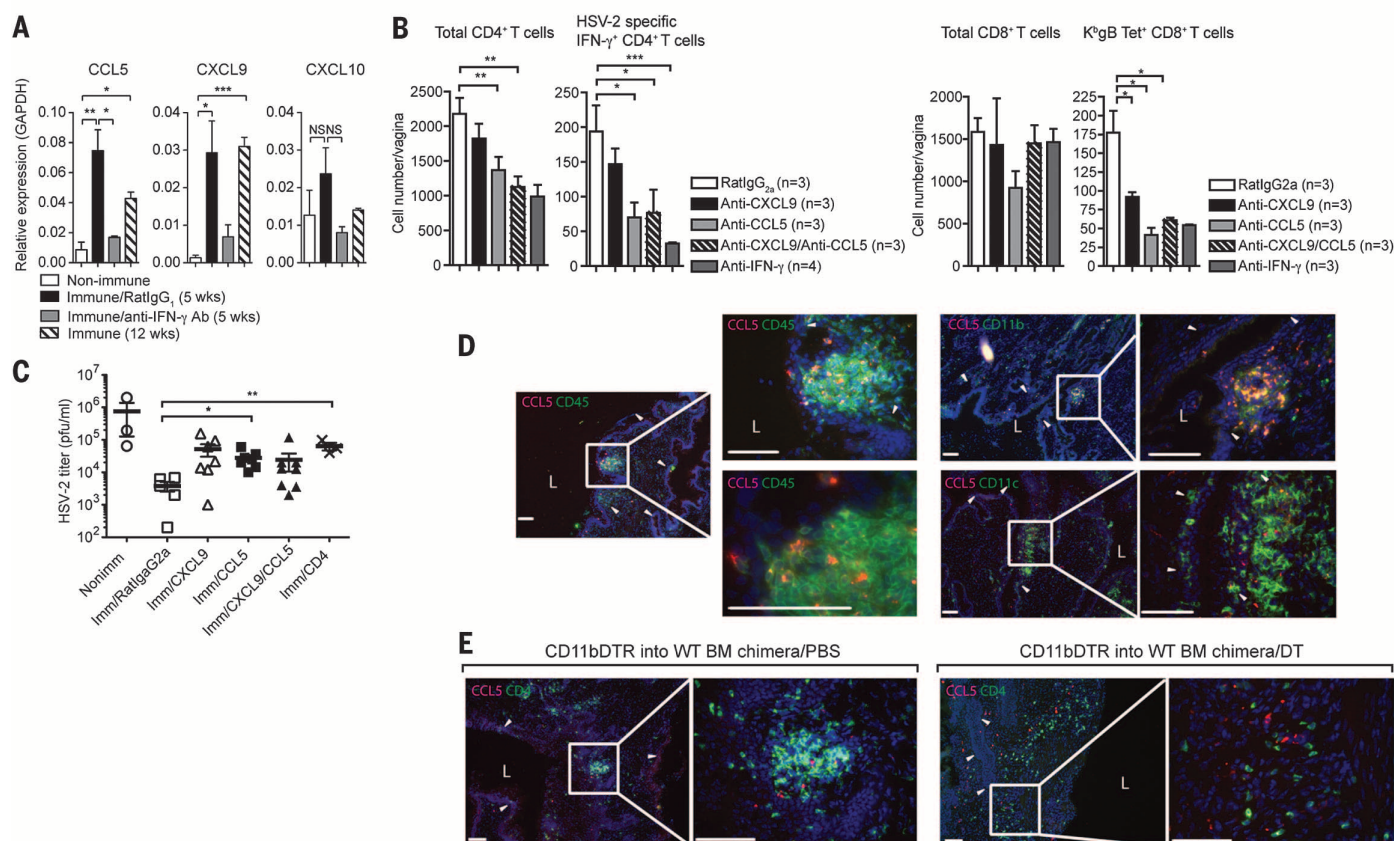


Fig. 4. The chemokine CCL5 is required for the retention of T_{RM} in vaginal tissues after TK⁻ HSV-2 immunization. (A) Expression levels of CCL5, CXCL9, and CXCL10 in vaginal tissues of C57BL/6 mice immunized with TK⁻ HSV-2 5 or 12 weeks before were analyzed by qRT-PCR. A group of mice immunized for 5 weeks was treated with rat IgG₁ (n = 5) or antibody against IFN- γ (R4-6A2) (n = 3) intravaginally for four consecutive days. mRNA expression of the indicated genes was normalized to that of GAPDH for each sample. (B) C57/BL6 mice intravaginally immunized with TK⁻ HSV-2 5 weeks before were treated with isotype control IgG and CXCL9- and/or CCL5-specific antibody intravaginally for four consecutive days. On day 5, HSV-2-specific IFN- γ ⁺ CD4⁺ T cells and tetramer⁺ CD8⁺ T cells in the vagina were analyzed by flow cytometry. (C) Mice immunized and treated as in (B)

were challenged with a lethal dose of WT HSV-2 intravaginally 1 day after the antibody treatment regimen. Virus titers in the vaginal wash were measured on day 1 after challenge. (D) Frozen sections of vaginal tissue from mice immunized with ivag TK⁻ HSV-2 5 weeks before were stained with antibodies against CCL5 (red) and CD45, CD11b, or CD11c (green). (E) CD11bDTR into WT bone marrow chimera immunized with TK⁻ HSV-2 5 weeks before were treated with diphtheria toxin, and frozen sections of vaginal tissue were stained with antibodies against CCL5 (red) and CD4 (green). Nuclei are depicted by 4',6'-diamidino-2-phenylindole (DAPI) stain (blue). Images were captured using a 10 $\times$ or 40 $\times$ objective lens. Scale bars indicate 100 μ m. These data are representative of three similar experiments. Data are means $\pm$ SEM. **P* < 0.05; ***P* < 0.001; ****P* < 0.001.

which suggests that this may be a common mechanism of adaptive immune defense in the genital mucosa. Our data strongly support the need for designing vaccines for sexually transmitted infections that establish local memory in the T cell pool.

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SUPPLEMENTARY MATERIALS

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Materials and Methods

Figs. S1 to S20

References (26–39)

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T CELL MEMORY

Resident memory CD8 T cells trigger protective innate and adaptive immune responses

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The pathogen recognition theory dictates that, upon viral infection, the innate immune system first detects microbial products and then responds by providing instructions to adaptive CD8 T cells. Here, we show in mice that tissue resident memory CD8 T cells (T_{RM} cells), non-recirculating cells located at common sites of infection, can achieve near-sterilizing immunity against viral infections by reversing this flow of information. Upon antigen resensitization within the mouse female reproductive mucosae, $CD8^+ T_{RM}$ cells secrete cytokines that trigger rapid adaptive and innate immune responses, including local humoral responses, maturation of local dendritic cells, and activation of natural killer cells. This provided near-sterilizing immunity against an antigenically unrelated viral infection. Thus, $CD8^+ T_{RM}$ cells rapidly trigger an antiviral state by amplifying receptor-derived signals from previously encountered pathogens.

CD8 T cells control viral infections. To be licensed with effector functions, naïve CD8 T cells must first be activated by specialized members of the innate immune system that have been alarmed by danger signals in the form of recognition of broadly conserved microbial associated molecular patterns [such as double-stranded RNA or lipopolysaccharide (LPS)] (1). Paradigmatically, $CD8^+ T$ cells act very locally by contacting infected host cells for both the detection of pathogen-associated peptides presented by major histocompatibility complex class I (MHC-I) and target-cell-specific delivery of toxic effector molecules (2). After clearance of infection, memory T cells remain. Central, effector, and resident memory T cell subsets (T_{CM} , T_{EM} ,

and T_{RM} , respectively) occupy different anatomic niches, where they fulfill distinct roles in protective immunity (3, 4). The most recently described subset, T_{RM} , comprises non-recirculating memory T cells that remain positioned at common portals of reinfection, including barrier tissues such as the mucosae and skin (5–10). When present at the site of reinfection, T_{RM} cells accelerate protection against homologous reinfections, although the mechanism remains obscure (10–15). We recently demonstrated that reactivation of T_{RM} cells results in bystander recruitment of recirculating memory T cells to the site of anamnestic Ag exposure in a manner dependent on the cytokine interferon- γ (IFN- γ) (16). This suggested that T_{RM} cells serve a sentinel and alarm function in addition to more prototypical CD8 T cell functions.

We wanted to further understand the mechanism by which $CD8^+ T_{RM}$ cells communicated local pathogen-associated antigen reencounter to recirculating memory CD8 T cells located outside the tissue. OT-I or P14 chimeric mice, which contained ovalbumin (OVA)-derived SIINFEKL (E,

Glu; F, Phe; I, Ile; K, Lys; L, Leu; N, Asn; S, Ser) or gp33 peptide-specific $CD8^+ T_{RM}$ cells, respectively, were established in the female reproductive tract (FRT) as follows: Naïve OT-I or P14 CD8 T cells were transferred intravenously to naïve C57BL/6J mice, and mice were infected 1 day later with recombinant vaccinia virus expressing OVA (VV-OVA) or lymphocytic choriomeningitis virus (LCMV), respectively (fig. S1, A and B). FRT T_{RM} cells were then reactivated locally by depositing cognate SIINFEKL or gp33 peptide into the cervical lumen (transcervical, t.c.), as described (17). We found that T_{RM} reactivation with either peptide or recombinant vaccinia virus expressing cognate peptide induced expression of the cell adhesion molecule VCAM-1 (vascular cell adhesion molecule-1) on local vascular endothelium (Fig. 1, A to C). IFN- γ is produced by reactivated T_{RM} cells in vivo (16). We found that exogenous t.c. deposition of IFN- γ into naïve mice was sufficient to induce local VCAM-1 expression (fig. S2). Further experiments revealed that when OT-I *Ifng*^{−/−} T_{RM} cells were reactivated in wild-type mice, VCAM-1 was no longer up-regulated, demonstrating that T_{RM} cells induce local VCAM-1 in a cell-autonomous manner by secreting IFN- γ upon antigen resensitization (Fig. 1, A and B). Additionally, when circulating P14 memory CD8 T cells were depleted (while preserving T_{RM} in the FRT) by injecting 1 μ g HIS51 (a Thy1.1-depleting antibody) (16), peptide reactivation resulted in similar levels of VCAM-1 on endothelial cells, suggesting that VCAM-1 expression is driven by local CD8 T cells (Fig. 1D).

VCAM-1 is the ligand for $\alpha 4\beta 1$ integrin, which plays a role in lymphocyte migration (18). Bystander OT-I CD8 T cell recruitment in response to gp33-specific T_{RM} reactivation was inhibited when either VCAM-1 or $\alpha 4\beta 1$ (CD49d) was blocked with neutralizing antibodies (Fig. 1E). Because $CD8^+ T_{RM}$ cells communicate antigen sensitization to recirculating CD8 T cells through an IFN- γ -VCAM-1 axis, we asked whether other lymphocyte lineages might also be recruited. B cells accumulated within the FRT within 12 hours of local $CD8^+ T_{RM}$ reactivation and increased >100-fold by 48 hours (Fig. 1, F and G). Similar results were observed when recombinant gp33-expressing vaccinia virus was

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used to reactivate T_{RM} instead of peptide (Fig. 1H) and in LCMV immune mice that did not receive a transfer of P14 CD8 T cells (Fig. 1I and fig. S1C). Moreover, depletion of recirculating P14 CD8 T cells with a Thy1.1-depleting antibody (which preserves P14 T_{RM} within the FRT) did not impair B cell recruitment upon t.c. gp33 peptide challenge, demonstrating that B cell recruitment was driven by locally reactivated CD8 T cells (Fig. 1J). However, B cell recruitment was critically dependent on local CD8 T cell-derived IFN- γ and VCAM-1 induction (Fig. 1, K and L).

These observations indicate that reactivated T_{RM} cells may broadcast detection of pathogen-associated peptides to other cell types through cytokines. Although most microbes are cleared by the innate immune system without priming adaptive responses, professional pathogens require adaptive immunity for clearance. Dendritic cells (DCs) are principal messengers between the innate and adaptive immune systems. Detection of broadly conserved microbial associated molecular patterns instructs DC maturation, which in turn initiates T cell activation (1, 19, 20). Because

T_{RM} recognition of peptides indicates reexposure to a pathogen that previously caused sufficient infection to elicit an adaptive immune response, we asked whether CD8 $^{+}$ T_{RM} reactivation could trigger DC maturation. When either transgenic or endogenous cognate-antigen-specific CD8 $^{+}$ T_{RM} cells were established, local exposure to either pathogen-associated peptide or recombinant VV expressing cognate peptide resulted in DC maturation within only 12 hours, as indicated by the induced expression of co-stimulatory molecules CD80, CD86, and CD40, as well as the lymph

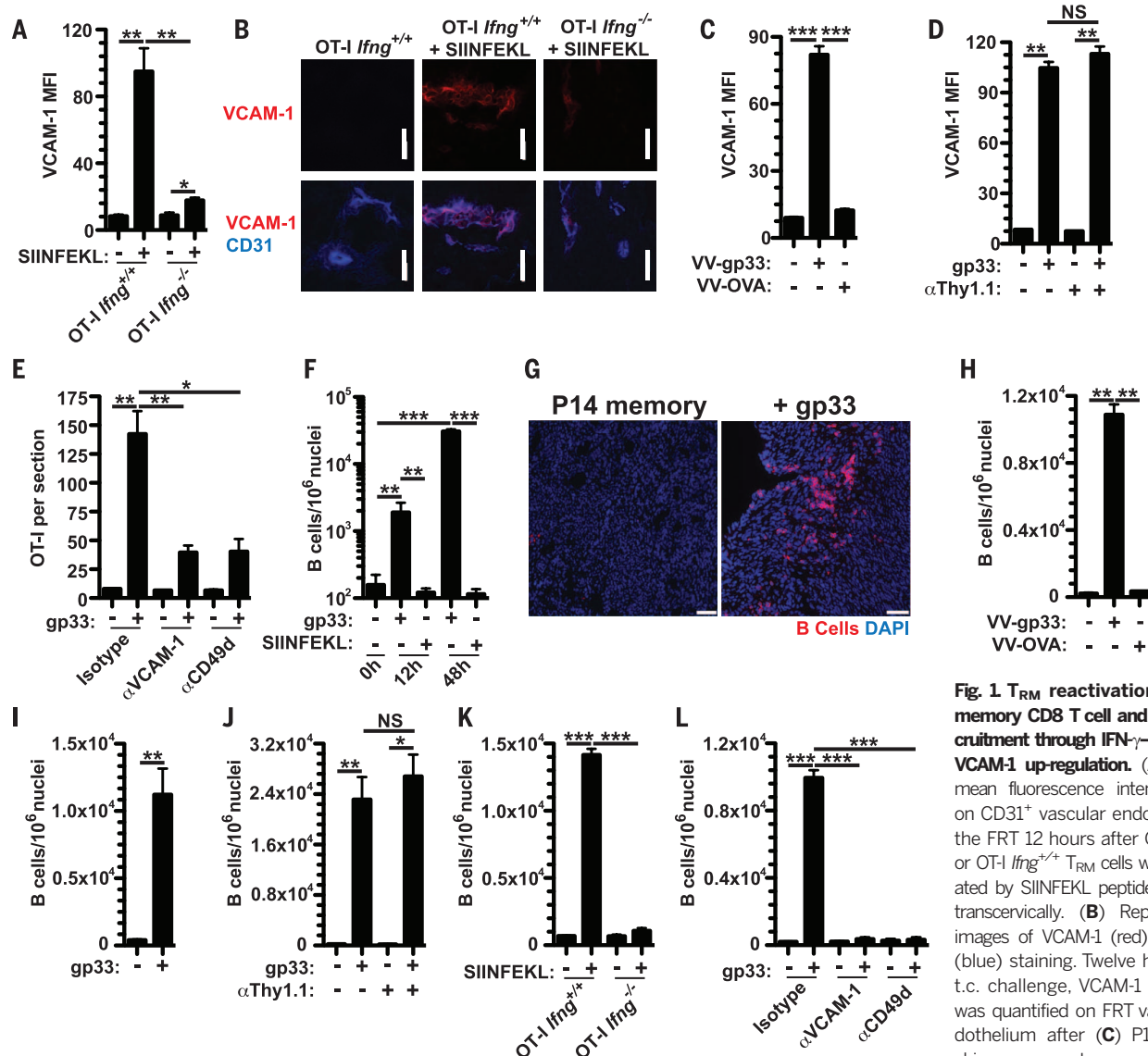


Fig. 1 T_{RM} reactivation induces memory CD8 T cell and B cell recruitment through IFN- γ -dependent VCAM-1 up-regulation. (A) VCAM-1 mean fluorescence intensity (MFI) on CD31 $^{+}$ vascular endothelium in the FRT 12 hours after OT-I *Ifng* $^{+/+}$ or OT-I *Ifng* $^{-/-}$ T_{RM} cells were reactivated by SIINFEKL peptide deposited transcutaneously. (B) Representative images of VCAM-1 (red) and CD31 (blue) staining. Twelve hours after t.c. challenge, VCAM-1 expression was quantified on FRT vascular endothelium after (C) P14 immune chimera were transcutaneously challenged with either VV-gp33 or VV-OVA, left untreated, or (D) when P14 immune chimera were injected with Thy1.1-depleting antibody or left untreated 5 days before t.c. deposition of gp33 peptide. (E) OT-I CD8 T cells were transferred intravenously into P14 immune chimera that were treated with antibodies against VCAM-1, CD49d, or isotype control and were then challenged with gp33 transcutaneously. OT-I T cells per coronal section were enumerated 48 hours later. (F) B cells in the FRT were enumerated 12 and 48 hours after t.c. gp33 peptide challenge of P14 immune chimera. (G) Representative images. DAPI, 4',6-diamidino-2-phenylindole. B cells in the FRT were also enumerated in (H) P14 immune chimera that were transcutaneously challenged with either VV-gp33 or VV-OVA or left untreated in (I) LCMV immune mice that never received P14 cells, and (J) in P14 immune chimera that were injected with Thy1.1-depleting antibody 5 days before t.c. gp33 peptide challenge. (K) B cells within the FRT of OT-I *Ifng* $^{+/+}$ or OT-I *Ifng* $^{-/-}$ immune chimera were enumerated 48 hours after t.c. SIINFEKL challenge. (L) B cells in the FRT were quantified 48 hours after P14 immune chimera were challenged transcutaneously with gp33 peptide in the presence of VCAM-1 or CD49d blocking antibodies. Each experiment shown includes three to six mice, and data are representative of two or three independent experiments. Scale bars indicate 20 μ m. NS, not significant; * P < 0.05, ** P < 0.01, *** P < 0.001; unpaired two-tailed t test; error bars indicate mean $\pm$ SEM.

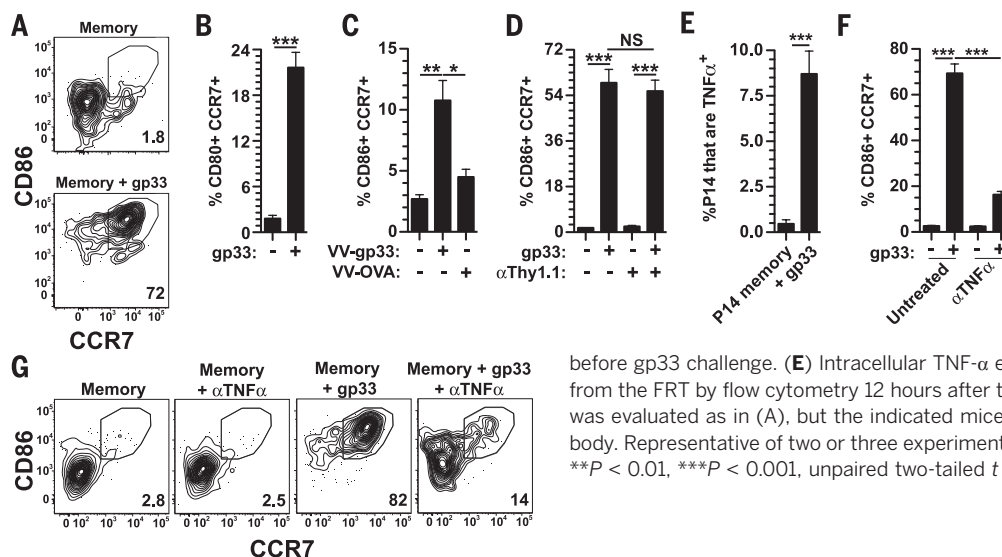


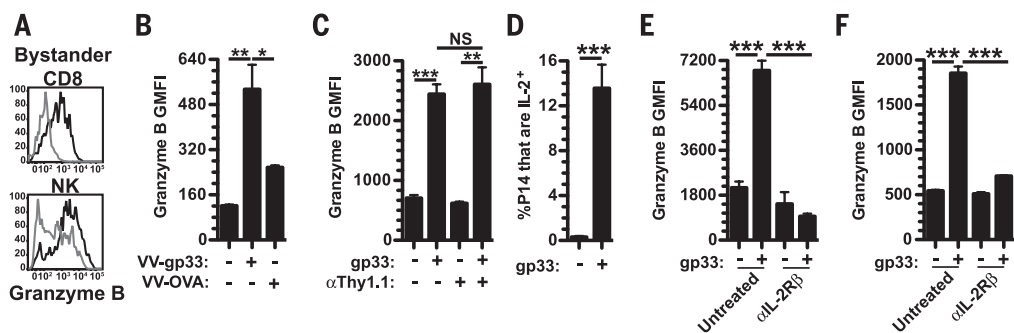
Fig. 2. T_{RM} reactivation induces DC maturation. CD86 or CD80 and CCR7 expression was evaluated on CD11c⁺/MHC-II⁺ DCs in the FRT 12h after t.c challenge of (A) P14 immune chimeras challenged with gp33 peptide, (B) LCMV immune mice (that never received P14 cells) challenged with gp33 peptide, (C) P14 immune chimeras that were transcritically challenged with either VV-gp33 or VV-OVA, or (D) P14 immune chimeras that were injected intraperitoneally with 1 μg of Thy1.1-depleting antibody 5 days

before gp33 challenge. (E) Intracellular TNF-α expression was evaluated in P14 CD8 T cells from the FRT by flow cytometry 12 hours after t.c. gp33 challenge. (F and G) DC phenotype was evaluated as in (A), but the indicated mice were pretreated with TNF-α-blocking antibody. Representative of two or three experiments totaling 6 to 14 mice per group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, unpaired two-tailed *t* test; error bars indicate mean ± SEM.

Fig. 3. T_{RM} reactivation induces NK cell activation.

(A) P14 immune chimeras were challenged transcritically with gp33 to reactivate T_{RM} cells. Twelve hours later, intracellular granzyme B expression was evaluated in bystander CD8 T cells (P14 CD8 T cells were excluded from analysis), and NK cells isolated from the FRT (gray line, without gp33 challenge; black, gp33 challenge). Intracellular granzyme B expression within NK cells isolated from the FRT was evaluated 12 hours after (B)

P14 immune chimeras were transcritically challenged with either VV-gp33 or VV-OVA or left untreated or (C) P14 immune chimeras were previously treated with Thy1.1-depleting antibody before t.c. gp33 peptide challenge. (D) Intracellular IL-2 expression by P14 CD8 T cells isolated from the FRT of P14 immune chimeras was evaluated 12 hours after t.c. gp33 peptide challenge. Intracellular



granzyme B expression by (E) NK cells and (F) bystander CD8 T cells isolated from the FRT of P14 chimeras 12 hours after gp33 peptide challenge when mice were pretreated with IL-2Rβ-blocking antibody. *n* = 3, representative of three experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, unpaired two-tailed *t* test, mean ± SEM. GMFI, geometric mean fluorescence intensity.

node homing chemokine receptor CCR7 (Fig. 2, A to C, and fig. S3). Depletion of circulating P14 CD8 T cells did not alter DC maturation after peptide rechallenge, suggesting that CD8 T_{RM} activation was sufficient for DC maturation (Fig. 2D). These data indicate that CD8⁺ T_{RM} ligands also serve as potent inducers of innate immune responses. Intracellular cytokine staining indicated that CD8⁺ T_{RM} cells expressed the cytokine tumor necrosis factor-α (TNF-α) in vivo within 12 hours of local reactivation (Fig. 2E) and that TNF-α was essential for DC maturation (Fig. 2, F and G).

Natural killer (NK) cells are innate lymphoid cells that play essential roles in control of viral infections (21, 22). They have advantages over the adaptive immune system because they are constitutively abundant and broadly distributed (21). When activated, they kill host cells showing signs of stress but lack highly specific antigen receptors for detecting pathogen-associated peptides (22). Thus, viral infections also require the presence of CD8 T cells for clearance. T_{RM} reactivation with either peptide or recombinant VV expressing cognate peptide resulted in granzyme B up-regulation among NK cells within the FRT within 12 hours, and this event also occurred when circulating mem-

ory P14 CD8 T cells were depleted (Fig. 3, A to C). Contemporaneously, local memory CD8 T cells of irrelevant specificities also increased expression of granzyme B (Fig. 3A). These data suggested that, in the event of local T_{RM} reactivation, NK cells and memory CD8 T cells receive signals to become poised for cytotoxicity of infected host cells (2). T_{RM} cells expressed the cytokine interleukin (IL)-2 in vivo within 12 hours of local reactivation (Fig. 3D), and blocking IL-2 receptor β (IL-2Rβ) abrogated granzyme B up-regulation on both NK cells and bystander memory CD8 T cells (Fig. 3, E and F).

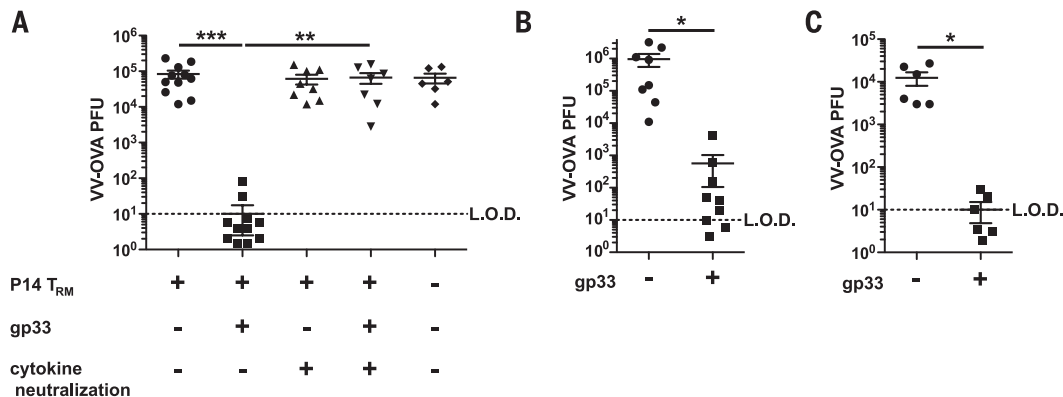
These data established that T_{RM} reactivation induced broad activation of innate and adaptive immune components. This observation provided impetus for the hypothesis that T_{RM} may protect the host in part by (i) acting as sensory cells for previously encountered peptides that are associated with intracellular pathogenic infections, (ii) communicating rechallenge to abundant nonspecific immune effectors, and thus (iii) triggering an organ-wide antiviral state. To test this hypothesis, we established P14 chimeric mice with gp33 peptide-specific T_{RM} cells within the FRT (Fig. 4). Mice were subsequently challenged with VV-OVA for which memory T cells had not been established.

As expected, infection resulted in abundant tissue viral load within 2 days of transcritic exposure in both naïve mice and in mice containing gp33-specific T_{RM} cells. However, if gp33 peptide was included in the inoculum in order to reactivate local T_{RM} cells at the time of viral challenge, we failed to detect infection in 9 of 11 mice, coinciding with a ~10⁴-fold reduction in viral load (Fig. 4A). Similar results were observed when gp33 peptide was delivered 12 hours before viral challenge (Fig. 4B) and in mice that harbored only endogenous gp33-specific T_{RM} cells (i.e., LCMV immune mice that did not receive a transfer of P14 CD8 T cells; Fig. 4C and fig. S1C). These data suggest that T_{RM} reactivation, which was associated with widespread activation of the local immune surveillance network, induced an antiviral state at the site of infection. This near sterilizing protection was completely abrogated when IFN-γ, TNF-α, and IL-2Rβ-dependent cytokines were blocked (Fig. 4A).

CD8 T cells are classically thought to control viral infections by contact-dependent interactions between antigen-specific CD8 T cells and each infected host cell, followed by directed target cell killing (2). These data support an expanded range of functions by which T_{RM} cells mediate protective

Fig. 4. T_{RM} reactivation induces antiviral state.

(A) P14 immune chimeras or control naïve mice were challenged transcutaneously with 4×10^6 plaque-forming units (PFU) of antigenically unrelated VV-OVA in the presence or absence of gp33-reactivating peptide and/or IFN- γ , TNF- α , and IL-2R β -blocking antibodies. Two days later, viral titers were evaluated by plaque assay from homogenized FRT. Data were pooled from two independent experiments totaling 6 to 11 mice per group. (B) As in (A), however, gp33 peptide was delivered 12 hours before viral challenge. Data pooled from two independent experiments totaling eight or nine mice per group. (C) LCMV immune mice (without P14 transfer) were transcutaneously challenged with 1×10^6 PFU of VV-OVA in the presence or absence of gp33



immunity. Here, we show that T_{RM} cells reverse the flow of information from innate to adaptive immune systems by demonstrating that adaptive T_{RM} sensitization initiates broad local immune activation. In this light, our data suggest that T_{RM} cells could be viewed as a pathogen recognition entity that engages innate immune functions more sensitively than what is accomplished by innate recognition of conserved microbial associated molecular patterns. The T_{RM} functions described here were dependent on IFN- γ , TNF- α , and IL-2R β -dependent cytokines, which may help explain why memory CD8 T cell populations that are competent to produce each of these cytokines (referred to as polyfunctional memory CD8 T cells) are often best associated with protective immunity (23).

It may be possible to leverage the functions of T_{RM} described here for therapeutic purpose. For instance, local reactivation of established T_{RM} cells with peptide vaccines could be used to increase local immunity against unrelated pathogens. The potent inflammatory effects of T_{RM} reactivation may also have pathological consequences and may contribute to the observed association between viral infections and exacerbation of tissue-specific autoimmune or inflammatory diseases. Principally, our results indicate how sensitization of relatively small numbers of T_{RM} cells may result in an amplified signal to more-abundant members of the innate immune system in order to trigger an organ-wide antiviral state.

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SUPPLEMENTARY MATERIALS

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Figs. S1 to S3
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T CELL MEMORY

Skin-resident memory CD8⁺ T cells trigger a state of tissue-wide pathogen alert

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After an infection, pathogen-specific tissue-resident memory T cells (T_{RM} cells) persist in nonlymphoid tissues to provide rapid control upon reinfection, and vaccination strategies that create T_{RM} cell pools at sites of pathogen entry are therefore attractive. However, it is not well understood how T_{RM} cells provide such pathogen protection. Here, we demonstrate that activated T_{RM} cells in mouse skin profoundly alter the local tissue environment by inducing a number of broadly active antiviral and antibacterial genes. This “pathogen alert” allows skin T_{RM} cells to protect against an antigenically unrelated virus. These data describe a mechanism by which tissue-resident memory CD8⁺ T cells protect previously infected sites that is rapid, amplifies the activation of a small number of cells into an organ-wide response, and has the capacity to control escape variants.

Tissue-resident memory CD8⁺ T cells (T_{RM} cells) are a subtype of memory lymphocytes (1) that permanently reside in nonlymphoid tissues in mice and humans (2–11). Analysis of herpes simplex virus (HSV)-1 and HSV-2

shedding episodes in infected human mucosa has shown that emerging lesions are often controlled within 6 to 12 hours (12). Furthermore, the severity of viral lesions during reactivation is likely determined by local immune control, and data in

mouse models suggest that such tissue protection can be mediated by locally residing memory CD8⁺ T cells (13–15).

The mechanisms by which a small number of local memory cells can protect a peripheral tissue have not been established and, given the low numbers of T_{RM} cells in tissues, unlikely to solely involve the direct killing of target cells (16). In addition, although T_{RM} cells are able to recruit circulating memory CD8⁺ T cells to the peripheral tissue within 48 hours of activation (17), such recruitment is unlikely to achieve early pathogen control (12).

To investigate how small numbers of tissue-resident memory T cells confer rapid protection of local tissue, we created a pool of T_{RM} cells by intra-epidermal DNA vaccination of mice that had received small numbers of green fluorescent protein CD8 T cells specific for the HSV-1-derived glycoprotein B peptide (gB₄₉₈₋₅₀₅ (gBT-GFP hereafter)) (fig. S1) (9, 18). Weeks later, skin areas harboring T_{RM} cells were challenged with HSV-1 or gB₄₉₈₋₅₀₅ peptide. Immunohistochemical analysis of HSV-1-infected or gB₄₉₈₋₅₀₅ peptide-challenged skin tissue of gBT-GFP T_{RM} cell mice and naive mice at 9 hours after infection/peptide administration did not reveal any difference in the infiltration of macrophages, gBT-GFP memory T cells, or CD3 cells. A moderate increase in the number of neutrophils was only observed upon peptide administration (fig. S2, A to F). To evaluate other possible effects of T_{RM} cell activation on the surrounding tissue, we obtained transcriptional profiles from the entire skin tissue at the same early time point after in situ triggering of T_{RM} cells. Comparison of the transcriptional profiles in skin exposed to control [ovalbumin (OVA₂₅₇₋₂₆₄)] peptide or cognate (gB₄₉₈₋₅₀₅) peptide revealed differential expression of a large number of genes [cut-offs: false discovery rate (FDR) < 0.05; log₂ fold change > ± 1.2] (Fig. 1A and fig. S3). To distinguish between noise caused by variation in tissue composition and signal due to T_{RM} cell triggering, transcriptional profiling was performed on a second cohort of mice (Fig. 1B and fig. S3). Genes the expression of which was altered at a comparable magnitude in both data sets (difference in magnitude of induction < 1.5; blue in fig. S3B) were retained for further analysis.

To determine whether the observed changes in gene expression were due to T cell receptor (TCR) recognition of antigen, cohorts of mice harboring T_{RM} cells specific for the OVA₂₅₇₋₂₆₄ epitope (OTI hereafter) were challenged with either gB₄₉₈₋₅₀₅ or OVA₂₅₇₋₂₆₄ antigen. In this setup, activation of the OTI-GFP T_{RM} cells by cognate OVA₂₅₇₋₂₆₄ resulted in a reproducible change of the skin transcriptional profile (Fig. 1,

C and D). Furthermore, changes in gene expression in gBT-GFP skin T_{RM} cells challenged with cognate gB₄₉₈₋₅₀₅ peptide and OTI-GFP T_{RM} cells challenged with cognate OVA₂₅₇₋₂₆₄ peptide were highly correlated (Fig. 1E). Thus, triggering of T_{RM} cells harboring skin with peptide antigen leads to a rapid alteration in the transcriptome that is visible at the level of the entire tissue before substantial influx of immune cells is seen.

Combination of all four data sets resulted in a list of 89 genes that are differentially expressed (all increased) upon specific triggering of T_{RM} cells (table S1 and Fig. 1E). Induction of part of this gene set was already observed 3 hours after antigen administration, and induction was essentially complete after 6 hours (fig. S4). Supporting the immunohistochemical results, T cell-specific genes did not show any significant difference between T_{RM} cells harboring skin treated with specific or control peptide (fig. S5). Independent full transcriptome gene ontology analyses (19) of the four data sets indicated inflammation and immunity as dominant signatures of all data sets (table S2).

Transcriptome analysis of two independent experiments in which gBT-GFP T_{RM} cell skin was challenged with HSV-1 or control showed a similar pattern of gene induction. For most genes within the gene set (table S1), the magnitude of induction was larger upon peptide triggering, possibly because a greater number of T_{RM} cells can encounter antigen early after peptide administration (group 1 in Fig. 1F). In addition, a second group of genes, including a large number of chemokines and cytokines involved in innate immune cell movement, was more strongly or only up-regulated upon virus infection (group 2 in Fig. 1F). Together, these data show that antigen-specific activation of T_{RM} cells is sufficient to initiate an early response that is visible at the level of the entire tissue.

Strikingly, many of the genes that were induced upon peptide administration were expressed at levels >10 to >100 times the level of T cell-specific genes (Fig. 1G). Furthermore, analysis of the identified gene set revealed the induction of a broad-spectrum antipathogen response. To dissect whether this rapid tissue response depends upon systemic antigen-specific memory T cells, or only requires the T_{RM} cell population, OTI-GFP cells from male donors were transferred into syngeneic female recipients and activated by vaccination. In this setting, the systemic memory T cell pool (central memory + effector memory cells; T_{CM} + T_{EM})—but not the tissue-resident memory pool—is cleared (5) (fig. S1). Comparison of the transcriptional profile in skin of recipients harboring either T_{RM} cells or both T_{RM} and T_{CM} + T_{EM} cells indicates that activation of the T_{RM} cell pool is sufficient to induce expression of the large series of genes within skin (Fig. 2A).

Upstream regulator analysis of the induced gene signatures by Ingenuity Pathway Analysis indicated that the cytokine interferon-γ (IFN-γ) is the most likely factor controlling the transcriptional alterations seen in T_{RM} cell-conditioned

skin (table S3), and previous work has shown that CD8⁺ T_{RM} cells rapidly re-express IFN-γ after local antigen rechallenge (17). Analysis of full-thickness skin 9 hours after triggering of a population of wild-type or *Ifng*^{-/-} T_{RM} cells revealed that a large part of the transcriptional alterations seen upon T_{RM} cell triggering are dependent on T_{RM} cell-derived IFN-γ (Fig. 2B). Furthermore, this IFN-γ acts on skin cells other than T_{RM} cells themselves, as the tissue response is also largely lost in *Ifngr*^{-/-} recipient mice in which only T_{RM} cells express IFN-γ receptor 1 (Fig. 2C). These data suggest that shortly after TCR triggering, activated T_{RM} cells express IFN-γ to enhance expression of proteins involved in pathogen control within the surrounding tissue. To test this hypothesis, we analyzed the expression pattern of IFITM3 (interferon-induced transmembrane protein 3; blue in Fig. 1G), a protein with broad-spectrum antiviral activity (20), and one of the transcripts induced by T_{RM} cell triggering (Fig. 1 and table S1). Within 6 hours of T_{RM} cell activation by cognate antigen, most epidermal and dermal cells expressed IFITM3, with maximal levels at 9 to 18 hours after conditioning (Fig. 3). By 36 hours, IFITM3 expression was largely restricted to the outer layers of the epidermis, indicating that local T_{RM} cell activation leads to a transient change in the skin transcriptome that is still visible in aging keratinocytes by the time newly formed keratinocytes have returned to steady state (Fig. 3B).

In most models of infection control by CD8⁺ T cells, both the initial T cell activation and the final output signal (e.g., cytotoxicity) are dependent on recognition of cognate antigen. In contrast, the above-described tissue conditioning by T_{RM} cells requires antigen as input signal but generates an output signal—the up-regulation of genes involved in broad-spectrum defense—that does not rely on antigen recognition, a mechanism reminiscent of that of effector CD4⁺ T cells (21). To test the potential relevance of this state of T_{RM} cell-induced “pathogen alert,” we analyzed whether T_{RM} cell activation could lead to control of an antigenically unrelated pathogen in vivo. Skin-resident OTI-GFP T_{RM} cells were activated by local injection of cognate peptide, and 9 hours later the same area was infected with antigenically unrelated HSV-1. At two time points after virus administration, progression of HSV-1 infections was scored microscopically (day 1) and macroscopically (day 3). As expected, disease progression in naive mice was not influenced by OVA₂₅₇₋₂₆₄ peptide administration (Fig. 4A). In contrast, in mice harboring skin OTI-GFP T_{RM} cells, application of cognate OVA₂₅₇₋₂₆₄ peptide resulted in a strong reduction in HSV-1 disease severity relative to control conditions (Fig. 4A). Analysis of HSV-1-challenged skin tissue by staining with antibody to HSV showed that OTI-GFP T_{RM} cell activation resulted in a substantial reduction of both tissue necrosis and lateral spreading of herpetic lesions (Fig. 4B). In line with this, viral DNA levels were reduced in skin of mice harboring activated OTI-GFP T_{RM} cells at the time of infection (Fig. 4C, *P* < 0.0001). Taken

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together, these data demonstrate that the tissue conditioning that is induced by T_{RM} cell activation leads to enhanced pathogen control that is independent on the antigenic identity of this pathogen.

Three aspects of T_{RM} cell-mediated tissue conditioning are noteworthy. First, tissue conditioning is almost immediate. This property is likely to be of major relevance as, at least in case of HSV-2, early immune control

is the major determinant of episode severity (14). Second, tissue conditioning forms an effective amplification system, in which activation of a rare cell type leads to a tissue-wide response. Third, T_{RM} cell-mediated tissue

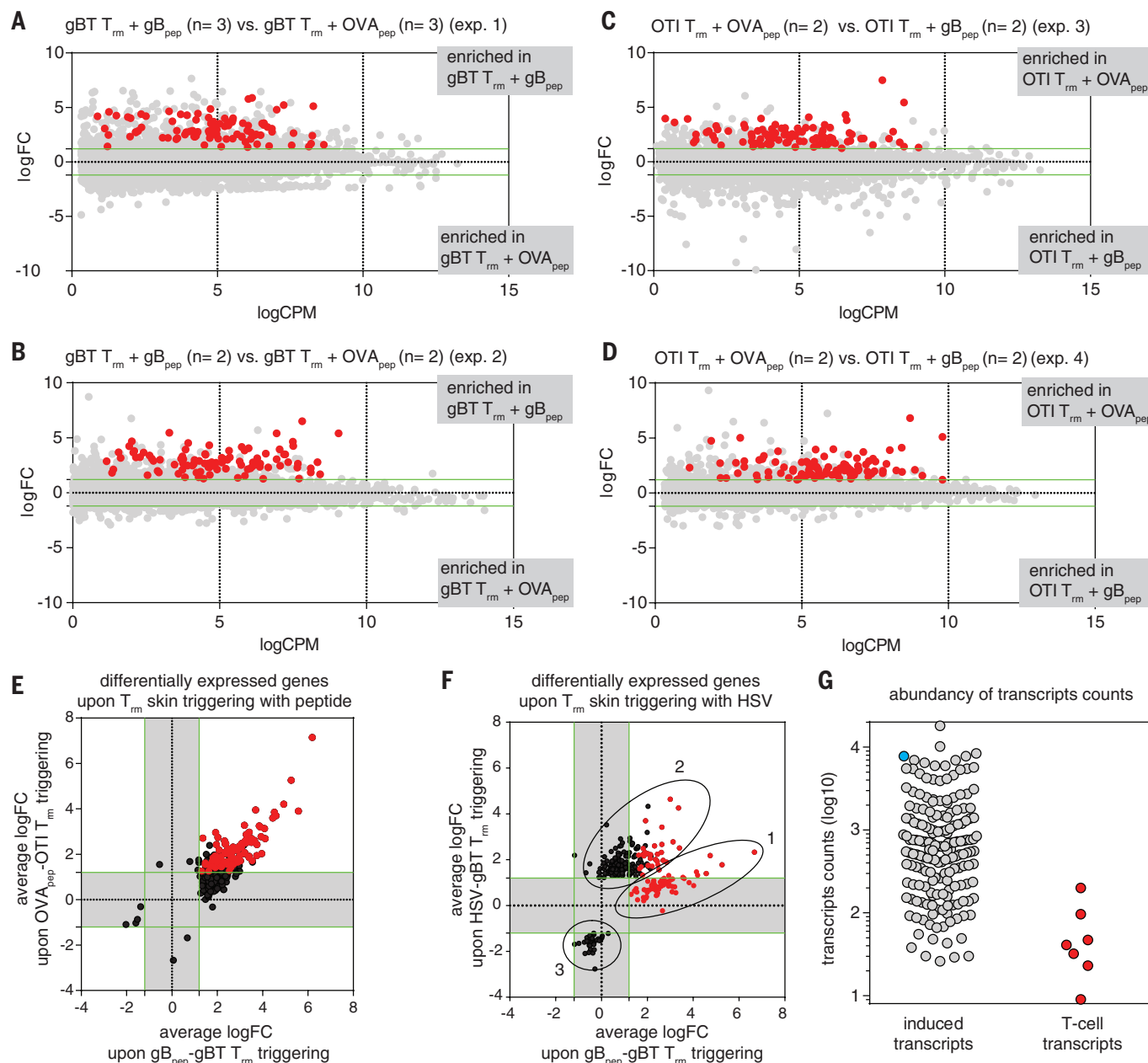


Fig. 1. T_{RM} cell triggering alters tissue-wide gene expression profiles. (A to D) Transcriptome analysis of full-thickness skin from mice harboring gBT-GFP T_{RM} cells (A and B) or OTI-GFP T_{RM} cells (C and D) upon local administration of either gB₄₉₈₋₅₀₅ or OVA₂₅₇₋₂₆₄ peptide. Relative abundance is plotted for averaged normalized read counts. All detected nondifferentially expressed genes are depicted in gray. Genes that are differentially expressed in all four comparisons ($\log$ fold change ($\log$ FC) $> \pm 1.2$; FDR < 0.05 ; difference in magnitude between replicate experiments < 1.5) (fig. S3) are depicted in red. In these and further plots, horizontal green lines represent $\log$ FC limits for significance ± 1.2 . (E) Average $\log$ FC for gBT T_{RM} cells and OTI T_{RM} cells harboring skin upon triggering with cognate peptide. Genes listed in table S1 (differentially expressed upon T_{RM} cell triggering) are shown in red; genes that

were only up-regulated in one of the T_{RM} cell groups in black. (F) Average $\log$ FC for skin harboring gBT T_{RM} cells upon triggering with either HSV-1 or cognate peptide. Genes listed in table S1 are shown in red; genes specifically up-regulated upon HSV-1 infection are depicted in black. Group 1, correlated behavior between both triggers, enriched in interferon-responsive genes; group 2, preferentially or only induced by HSV, enriched in secreted molecules; group 3, reduced by HSV, too small for pathway analysis. (G) Comparison of normalized transcript counts of the differentially expressed gene set in table S1 (induced transcripts) with normalized transcript counts of a set of T cell-specific genes (T cell transcripts). Among induced transcripts, IFITM3 is depicted in blue; the T cell transcripts gene set (IFN- γ , CD2, zap70, CD5, CD69, CD8a, and CD8b1) is depicted in red. Values are representative of eight comparisons.

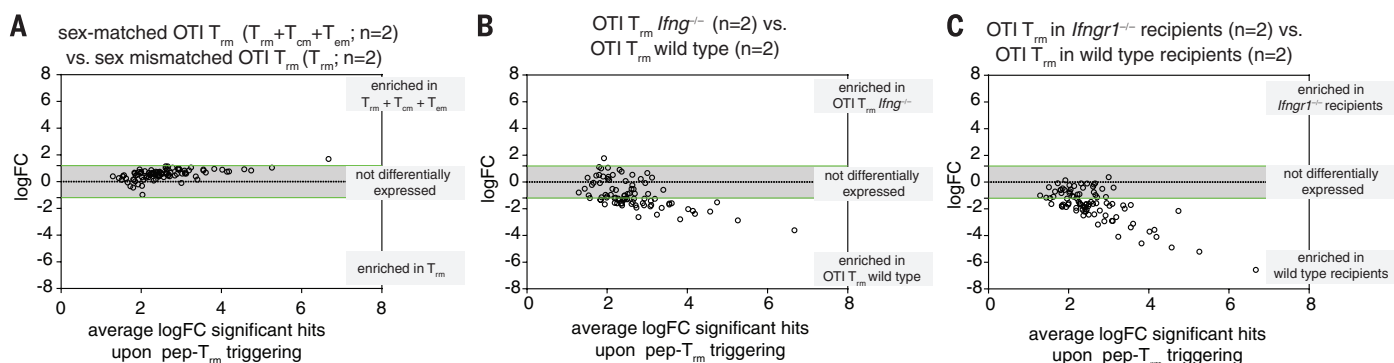


Fig. 2. T_{RM} cells mediate the induction of an antiviral state through IFN- γ .

(A) Female recipients of either sex-matched or mismatched OTI-GFP⁺ cells were vaccinated to induce skin T_{RM} cells. Skin areas harboring T_{RM} cells were treated locally with OVA₂₅₇₋₂₆₄ peptide and sacrificed 9 hours later. The absence of systemic memory T cells (see fig. S1) does not significantly reduce tissue conditioning by peptide triggering. On average, induction of the identified gene set was slightly more pronounced in sex-matched recipients (by a factor of 1.15, not

significant), which could either reflect a limited contribution of the circulating memory T cell pool or the slight reduction in T_{RM} cell numbers in skin of mismatched recipients (fig. S1F). (B) Recipients of OTI-GFP⁺ T_{RM} cells derived from either wild-type or IFN- γ -deficient donors were vaccinated to induce skin T_{RM} cells, and the effect of T_{RM} cell triggering was then analyzed as in (A). (C) IFN- γ -receptor-proficient or -deficient recipients of OTI-GFP⁺ T_{RM} cells were vaccinated to induce skin T_{RM} cells, and the effect of T_{RM} cell triggering was then analyzed as in (A).

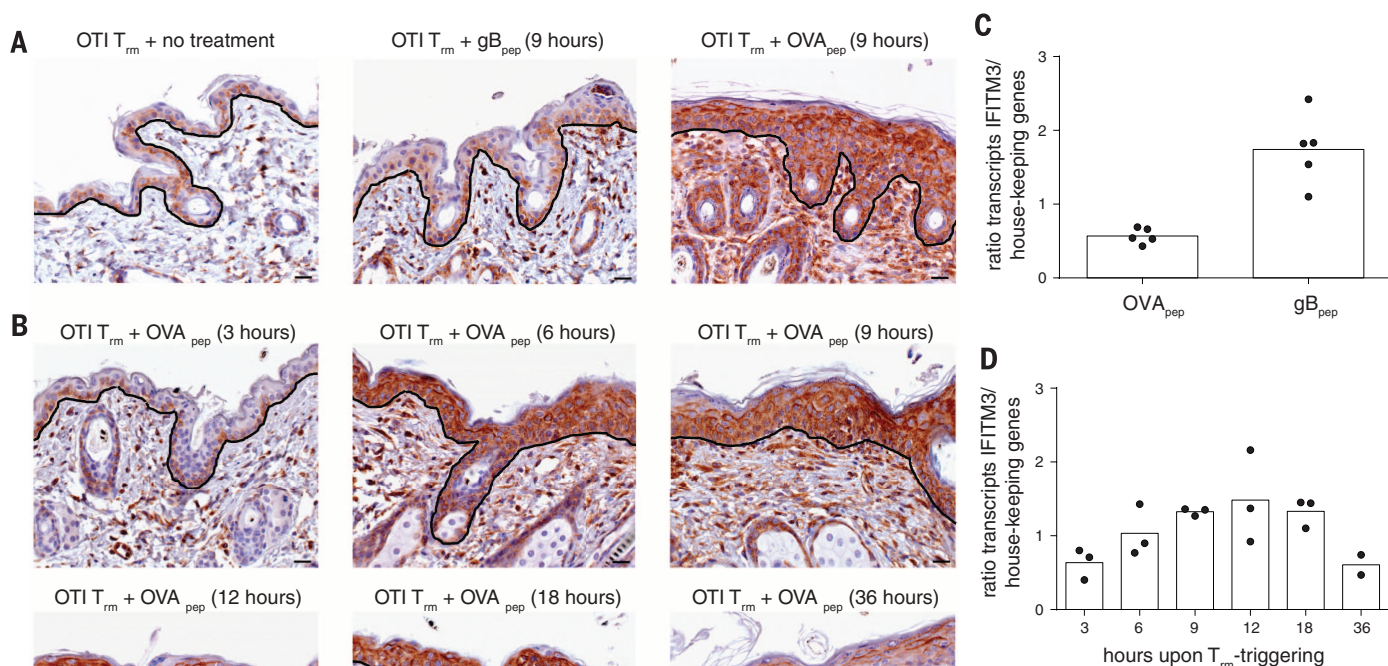


Fig. 3. Tissue conditioning by T_{RM} cells results in the induction of an antiviral state in large numbers of surrounding cells.

(A) Immunohistochemical detection of IFITM3 in the skin of mice harboring OTI-GFP⁺ T_{RM} cells, analyzed at steady state or 9 hours after treatment with OVA₂₅₇₋₂₆₄ peptide (representative of three mice per group). (B) Immunohistochemical detection of IFITM3 in the skin of mice harboring OTI-GFP⁺ T_{RM} cells at the indicated time points after treatment with OVA₂₅₇₋₂₆₄ peptide (representative of three mice per time point). The boundary between epidermis (top in all images) and dermis is highlighted by a black line. Scale bar, 20 μ m. (C and D) Naive gBT-GFP⁺ cells were transferred into recipients that were subsequently tattoo-vaccinated with DNA encoding TTFC-gB_{pep} to create a population of resident T_{RM} cells. Several weeks after tattooing, skin harboring T_{RM} cells was injected with either gB_{pep} or OVA_{pep} and processed for transcriptome analysis 9 hours later. (C) Ratio between IFITM3 counts and the median counts of 20 housekeeping genes. (D) The same analysis is depicted for samples in which skin harboring OTI T_{RM} cells was injected with OVA_{pep} and analyzed at the indicated time points.

conditioning results in protection that is ultimately antigen independent: Although initial T_{RM} cell activation requires recognition of antigen, the genes that are up-regulated in re-

sponse display activity toward a wide array of pathogens.

From a conceptual point of view, these data place T_{RM} cells as a bridge between the adaptive

and innate immune system, in which the TCR in T_{RM} cells has a function similar to that of Toll-like receptors in innate immune cells. From a practical point of view, the fact that T_{RM} cell

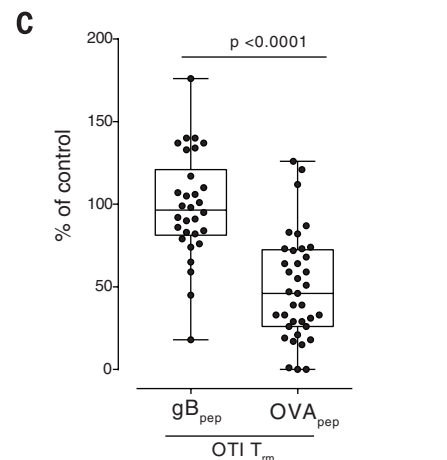
Fig. 4. CD8⁺ T_{RM} cell triggering provides cross-protection against an antigenically unrelated pathogen. (A) Naïve mice or mice harboring OTI-GFP⁺ T_{RM} cells (both hind legs) were injected locally with phosphate-buffered saline (PBS), cognate OVA₂₅₇₋₂₆₄ peptide and control gB₄₉₈₋₅₀₅ peptide and locally infected with HSV-1 9 hours later. Sixty hours after infection, the extent of each infection was scored by visual inspection by an observer blinded to experimental group (all naïve groups: *n* = 2; OTI-T_{RM} + PBS: *n* = 5; T_{RM} + gB₄₉₈₋₅₀₅: *n* = 10; T_{RM} + OVA₂₅₇₋₂₆₄: *n* = 13; both legs analyzed separately). (B) Mice harboring OTI-GFP⁺ T_{RM} cells (both hind legs) were injected locally with cognate OVA₂₅₇₋₂₆₄ peptide and control gB₄₉₈₋₅₀₅ peptide and were locally infected with HSV-1 9 hours

later. After 60 hours, the amount of viral DNA in infected skin was measured. Data are representative of four independent experiments with at least five mice per group. To allow comparison between experiments, the amount of viral DNA in the OTI-T_{RM} + gB₄₉₈₋₅₀₅ group was set to 100% for each experiment. (C) Immunohistochemical detection of HSV-1 infection in naïve mice

triggering leads to an output signal that no longer requires antigen recognition may also help counter-act viral escape. Recently, strategies have been put forward to create T_{RM} cell populations at sites of potential pathogen entry (10, 22, 23). The current data not only help to provide a mechanistic explanation for the effects of such vaccines but also suggest that in case of pathogens that exist as quasispecies, protection may conceivably be provided not only against the vaccine-encoded sequence but also against viral variants that are transferred in parallel.

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that received a local injection with OVA₂₅₇₋₂₆₄ peptide (B) or mice harboring OTI-GFP T_{RM} cells triggered with cognate OVA₂₅₇₋₂₆₄ peptide, control gB₄₉₈₋₅₀₅ peptide, or PBS (C). For each condition, two different magnifications of the same sample are shown. Data are representative of 2 (naïve group), 5 (T_{RM} + PBS), 10 (T_{RM} + gB₄₉₈₋₅₀₅), and 13 (T_{RM} + OVA₂₅₇₋₂₆₄) mice per group.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/346/6205/101/suppl/DC1
Materials and Methods
Figs. S1 to S5
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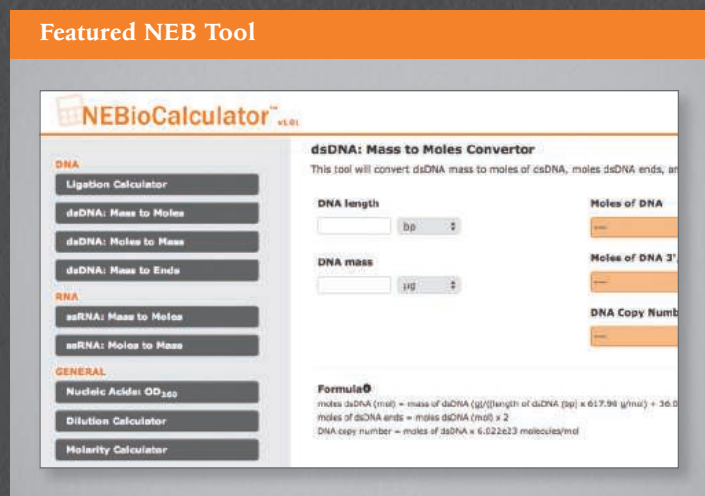


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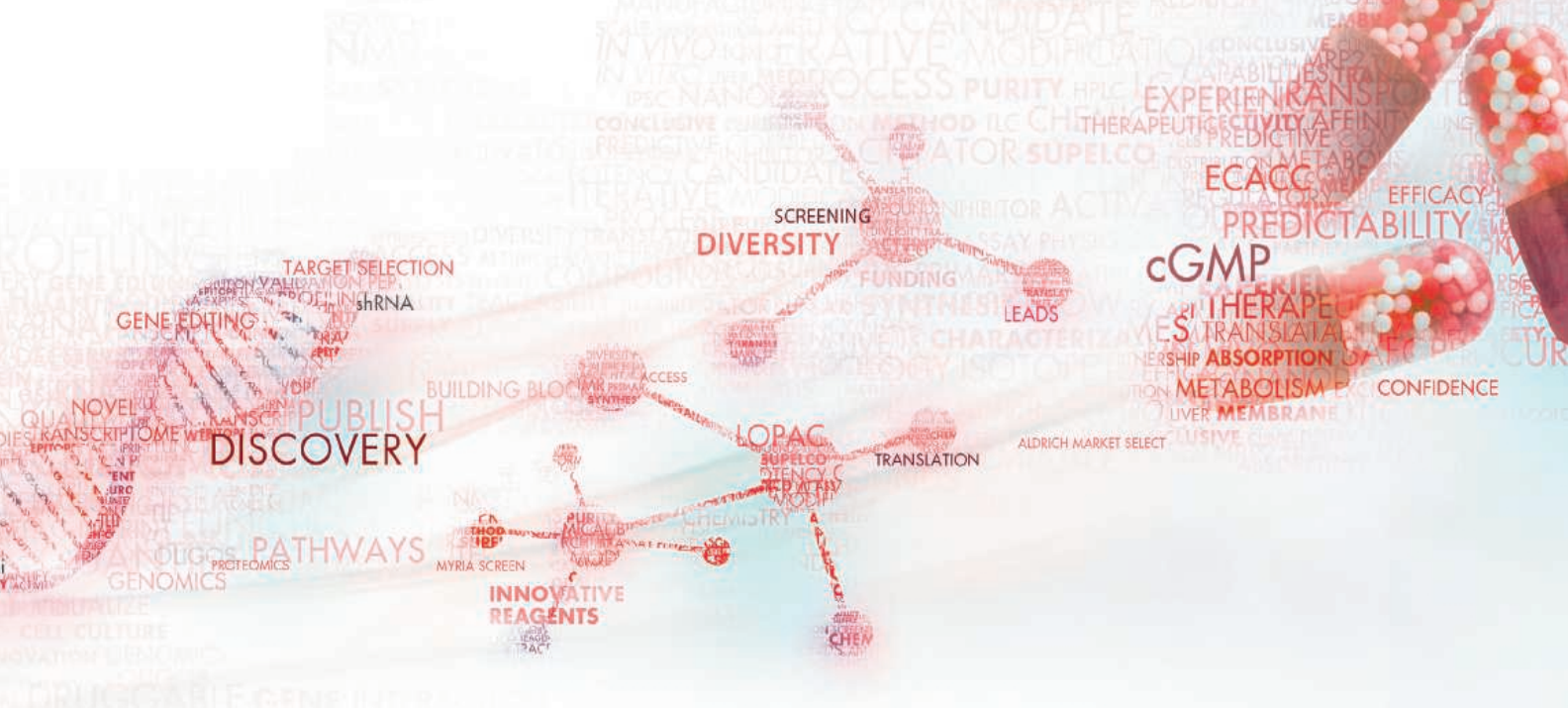
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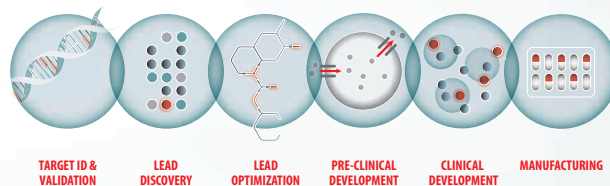
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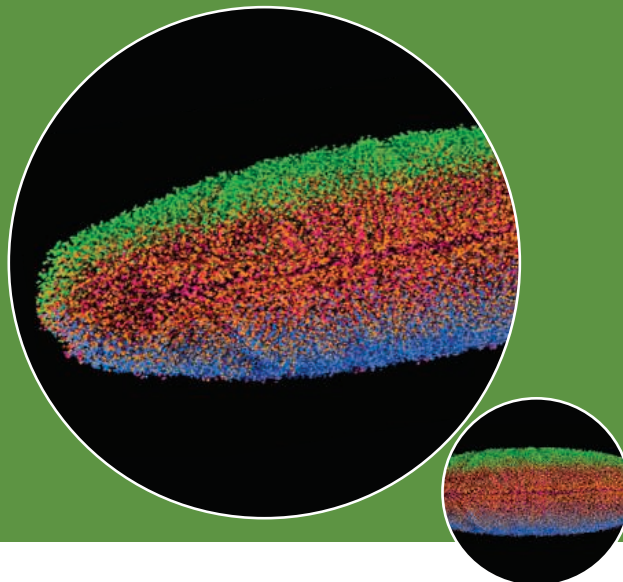
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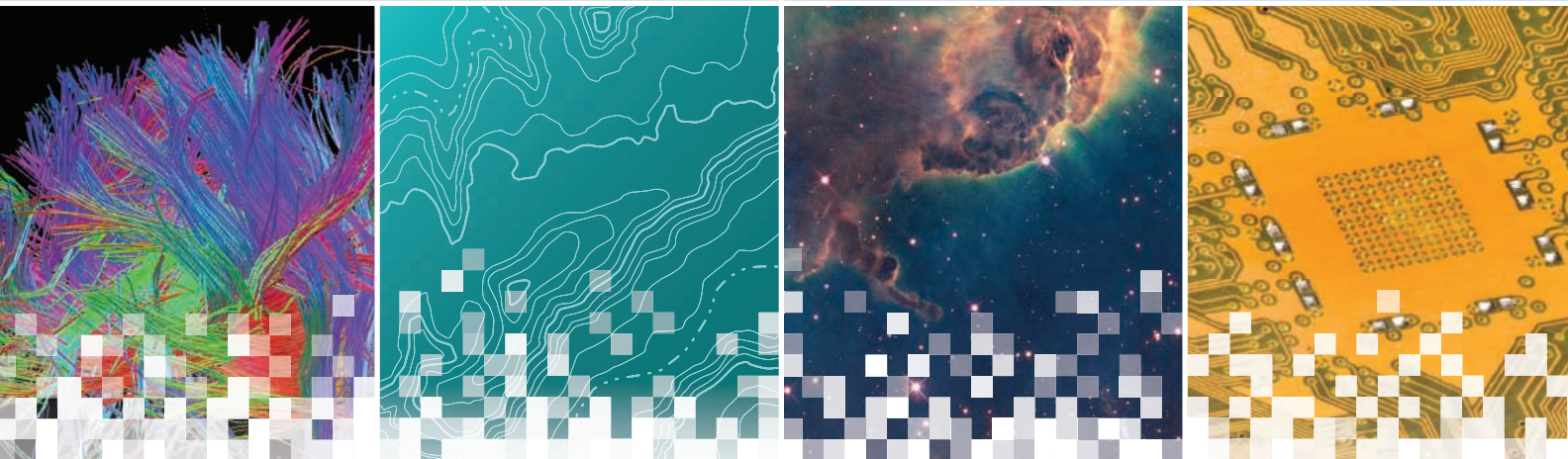
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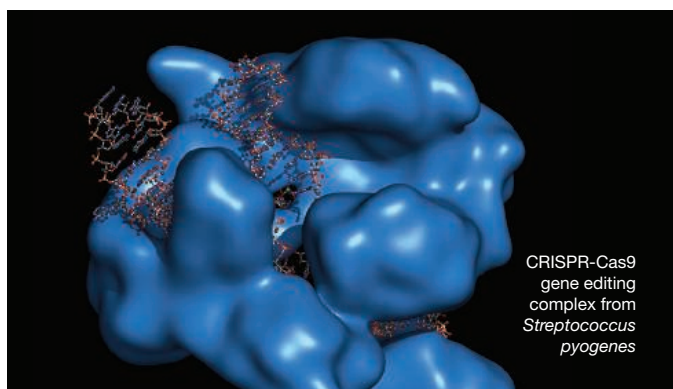
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Rewriting the Genome: Even DNA Needs an Editor

Until relatively recently, the power of molecular biology was at once vast and limited. Researchers who wanted to knock out specific genes to see what they did mostly had to restrict such studies to mice, and specific strains at that. Now a new class of genome- and epigenome-editing tools is reshaping the landscape. From *Arabidopsis* to humans to zebrafish, researchers are finding that, generally speaking, when it comes to the genome, if they can dream it, they can build it. **By Jeffrey M. Perkel**

To get a sense of the transformative power of genome-editing tools, consider plants. Unlike mice, there is no such thing as a plant embryonic stem (ES) cell. Even if there were, homologous recombination rates in plant cells are too low to yield reliable genetic modification without a little help. Thus, genetic tinkering in these organisms is typically accomplished using either random mutagenesis or genetic crosses, or via *Agrobacterium* infection or a specialized “gene gun.”

Such techniques have been used successfully, of course—most crop plants these days are genetically modified. But those efforts rely on random events; plant researchers had no way to make targeted genomic modifications, explains Dan Voytas, professor of genetics and director of the Center for Genome Engineering at the **University of Minnesota**, Minneapolis.

Now, thanks to genome engineering technology, they do. “You can say, okay, I know what that gene is, I know the sequence variation I need to introduce into my crop plants, and you can just go ahead and introduce it directly without engaging in an extensive breeding program,” Voytas says.

On the research front, such tools are nothing short of trans-

formative. Voytas uses them to rewrite the genome in plants such as tobacco and tomato, and they hold similar promise for other model organisms. In the clinic, genome editing can be coupled with human induced pluripotent stem cell technology to, for instance, create genetically repaired patient-specific transplants.

Questions of targeting specificity remain, but Phillip Sharp, institute professor at the Koch Institute for Integrative Cancer Research at the **Massachusetts Institute of Technology** (MIT), calls these technologies a “game-changer” on par with RNA interference. “RNAi was a major transition in how we do cell biology; this will be something comparable. And it’s going to greatly accelerate the speed in which we can probe problems from cancer to other normal development using our biological systems. It’s really a very important advance.”

Nuclease-based strategies

Genome-engineering approaches generally use a site-specific endonuclease to introduce a double-stranded DNA break at a specified point in the genome, like a custom restriction enzyme. As the cell repairs the break, it can either inadvertently disrupt the gene by adding or removing a few bases (a process called nonhomologous end-joining, or NHEJ) or use an exogenous piece of donor DNA to rewrite the damaged sequence to researchers’ specifications via homologous recombination.

One of the earliest nuclease technologies involved enzymes called meganucleases, but these were less than user-friendly, explains Philippe Duchateau, chief scientific officer at **Collectis**, a French genome-engineering firm that still uses the technology. “They are very difficult to engineer ... [requiring] a long and costly process.”

Zinc finger transcription factors have proven easier to manipulate. Researchers have long known that these proteins bind to DNA in a modular fashion, with each “finger” recognizing three to four specific nucleotides. In 2003, two teams independently demonstrated that they could string together custom arrays of zinc fingers to target novel DNA sequences and couple the array to the FokI nuclease to induce specific DNA changes at those sites. One group knocked out the *yellow* gene in fruit flies; the other repaired a GFP mutation in human cells. Two years later, researchers at **Sangamo BioSciences** used these so-called zinc finger nucleases (ZFNs) to repair a mutation in the IL2R-gamma gene in human cells, demonstrating the clinical potential of the technology.

Yet ZFNs never really took off in academia, in part because the technology was largely inaccessible—Sangamo owned the intellectual property, though they licensed it to **Sigma Aldrich** in 2007 for research purposes—and partly because good ZFNs are hard to build. In theory, explains Philip Gregory, senior vice president and chief scientific officer at Sangamo BioSciences, a library of 64 zinc fingers (one for each codon) should be sufficient to target any sequence. But in practice, a given finger’s binding properties differ from

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array to array. “Context matters,” he says.

In 2009, researchers deciphered a new class of transcription factors called TALEs. Like ZFNs, TALEs are modular structures, but each TALE module specifies a single nucleotide in the recognition sequence, and does so more or less independently of its neighbors. By fusing custom TALEs to the FokI nuclease (a so-called TALE nuclease, or TALEN), researchers could target pretty much any sequence they desired—though as TALENs are substantially larger than ZFNs and highly repetitive, the cloning process itself isn’t trivial (TALEN research tools are available through **Addgene** and **Life Technologies**, part of Thermo Fisher Scientific.)

TALENs made genome-editing technology accessible in a way it hadn’t been before. But in 2012 the editing landscape tilted on its axis again when Jennifer Doudna, a Howard Hughes Medical Institute (HHMI) investigator and professor of biochemistry and molecular biology at the **University of California, Berkeley**, and Emmanuelle Charpentier, then at **Umeå University**, worked out the mechanics of an RNA-guided bacterial immune system complex called clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9.

With reagents available through Addgene, **Horizon Discovery**, Life Technologies, **New England Biolabs**, and Sigma Aldrich, the two-component CRISPR-Cas9 is by far the simplest genome-editing system to date. It takes at least a week to make a TALEN, and sometimes months for a ZFN. But all CRISPR-Cas9 requires is a 20-nucleotide “single-guide” RNA specifying the desired target and Cas9 nuclease to cut it. That makes it easy for neophytes to implement the technology, and to explore different targeting sites. And, because its sequence targeting is encoded in RNA rather than protein, Cas9 can hit multiple sites at once, a process known as multiplexing.

Voytas likens genome-editing approaches to the evolution of DNA sequencing. “ZFNs are sort of [like] Maxam-Gilbert—you can get it to work and you can get the information, but you struggled to do so. TALENs are [like] Sanger sequencing. And next-generation sequencing is [akin to] CRISPR-Cas, it just happens so fast and easily,” he says.

Indeed, William Skarnes, a senior group leader at the **Wellcome Trust Sanger Institute** in Cambridge, United Kingdom, has spent more than a decade knocking out genes in mice the old-fashioned way. Between creating the targeting vectors, inserting them into ES cells, testing for gene modification, inserting the cells into blastocysts, and testing and crossing founders, each line could take from six to 12 months to create, he says. Now, that library is looking dated. “With CRISPR technology it’s just a simple injection of some very simple reagents directly in a one-cell embryo to create the same mutations. It’s remarkable—I mean, it’s something that we dreamt about at the very beginning of ES cell technologies.”

Targeting specificity

That’s not to say CRISPR-Cas9 is without flaws. At least in its initial incarnation, the system is less discriminating than users would like, and single-guide RNAs have been shown to induce

off-target effects in the human genome. Naturally, researchers have been developing strategies to counteract that problem.

Some, like **Harvard University** geneticist George Church, have shown that knocking out one of the two DNA cutting sites in Cas9 to create a so-called nickase, and pairing those molecules using two closely spaced guide RNAs, dramatically improves specificity. (That strategy is being commercialized by Sigma Aldrich, according to principal R&D scientist Greg Davis, who notes the approach works with guides as far as 150 bases apart. “This is a key distinguishing point,” he says, as it provides considerable targeting flexibility.) J. Keith Joung, associate chief of pathology for research at **Massachusetts General Hospital**, and David Liu at Harvard University independently described another approach, fusing a catalytically inactive Cas9 to FokI. Because FokI is an obligate dimer, that construction, like paired nickses, requires two binding events and thus yields greater precision, albeit with less targeting flexibility.

Guide RNA length can also influence specificity. Some have shown that extending the guide RNA by two nucleotides reduces off-target effects, while in Joung’s hands truncating the guide RNA reduces off-target mutation rates by up to 5,000 fold.

What is lacking, says Joung, is a side-by-side comparison to see which strategy truly performs best, not to mention a method—other than whole-genome sequencing—to identify modified sites across the genome. Still, Church says the technology—with an error rate of about one in 100 billion base pairs—probably is already sufficiently specific for research applications, where simple expedients such as comparing the effects of multiple guide RNAs can likely overcome possible off-target effects. Rather, it is in clinical applications where specificity really counts, he says, and the only way to determine if that’s an issue is to put the technology into animals, and ultimately humans, and see what happens.

“In the end it’s not going to be determined by sequencing off-targets,” he says. “It’s going to be determined by whether animals get cancer or not.”

Clinical applications

In early 2014, Sangamo published the first human clinical trial to use genome-editing technology. The company’s researchers harvested CD4⁺ T cells from HIV⁺ patients, infected them with a virus expressing a ZFN to knock out the HIV co-receptor CCR5 via NHEJ, and returned them to patients. “In every case, on infusion of the CD4 T cells, we see a marked **continued>**



Genome-engineering approaches generally use a site-specific endonuclease to introduce a double-stranded DNA break at a specified point in the genome.

increased in CD4 T cells in these patients,” says Gregory. Furthermore, those cells exhibited a “selective survival advantage” relative to unmodified cells, he says.

Sangamo is also pursuing homologous recombination-based therapeutics, says Gregory. For instance, its researchers recently published data demonstrating the ability to repair the IL2R-gamma gene in hematopoietic stem cells from patients with X-linked severe combined immunodeficiency—a cell population that theoretically could be used to reseed a patient’s bone marrow.

TALENs and CRISPRs also are heading to the clinic, with Collectis pursuing the former and **Editas Medicine** and **CRISPR Therapeutics** the latter.

In June, Daniel Anderson and Tyler Jacks at MIT demonstrated the potential clinical power of CRISPR-Cas9-based editing by delivering a vector co-expressing Cas9 and a guide RNA, plus a second DNA containing a repair template, into the tail veins of a mouse model of hereditary tyrosinemia type I, which is caused by a single point mutation in the fumarylacetoacetate hydrolase gene. Injection corrected the mutation in 1 out of every 250 liver cells, rescuing the diseased phenotype within a month. “I thought that was just amazing,” Doudna says.

Epigenome editing

Still, for all its potential as a nuclease, Cas9 can do so much more. The protein is just a “molecular machine that recognizes DNA in a targeted way,” Doudna says. As a result, it, and TALEs and zinc fingers for that matter, can serve as platforms for more extensive genomic tinkering—“epigenome engineering,” as it were.

Researchers have coupled catalytically inactive Cas9 and TALE domains to transcription activators and repressors, DNA methyltransferases, and histone modifying enzymes, thereby directing those activities to specific sites in the genome. They have coupled TALENs and Cas9 to fluorescent proteins to investigate chromosomal architecture. Church’s team has even identified Cas9 orthologs with different sequence requirements, which could be used, among other things, to target distinct activities to different genomic loci simultaneously.

Such epigenomic designs have potential clinical value, says Feng Zhang, core member of the Broad Institute of MIT and

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Harvard and a co-founder of Editas. “From a therapeutic perspective you may want to turn a gene on that needs to be on but for some epigenetic reason is turned off,” he says. For example, there are inherited diseases in which the maternal (expressed) copy of a gene is mutated while the paternal copy is wild type but silent. “One way to treat it might be to turn that gene on by using a Cas9 or TALEN activator,” Zhang says.

But such strategies present their own complications, especially regarding off-target effects. In one recent study, a team led by Sharp and Zhang used chromatin immunoprecipitation and next generation DNA sequencing (ChIP-Seq) to determine where in the genome a catalytically inactive but RNA-guided Cas9 protein binds. The team identified between 2,000 and 20,000 binding sites per guide RNA tested. But when they used a catalytically active Cas9, only one of 295 potential off-target sites the team tested was actually modified, suggesting that sequence binding and cutting occur in two distinct steps.

“This is a non-trivial nuance,” explains Stephen Ekker, professor of biochemistry and molecular biology at the **Mayo Clinic Cancer Center** in Rochester, Minnesota. “There are many reviews that argue that what happens is that the guide RNA finds double-stranded DNA in an extended complex, like a PCR primer does, and then Cas9 comes in and cuts it. That’s not how it works.” Sharp’s data instead suggest a genome-scanning mechanism in which binding and cutting are distinct events—an observation that implies epigenetic control using dead Cas9 fusions could have unintended consequences, as the protein may bind sites other than its intended targets. “Basically, any approach that really depends on having a single specific binding event may be compromised,” he says.

Still, there’s no denying the power of CRISPR-Cas9, and other editing tools. Ekker, who teaches a course on genome engineering, says molecular biology could be on the cusp of a technology boom akin to that ushered in by the invention of the transistor. Only time will tell what novel applications arise, he says. But this at least is clear: Researchers need no longer think of the genome as something with which they “tinker,” they can, well, engineer it.

Jeffrey M. Perkel is a freelance science writer based in Pocatello, Idaho.
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The University of Oklahoma

DIRECTOR OKLAHOMA GEOLOGICAL SURVEY

The Oklahoma Geological Survey (OGS) seeks applications for an exceptional, dynamic and visionary leader to serve as the 8th Director in its 106-year history. Located on the University of Oklahoma campus in Norman, Oklahoma, the OGS is a key public research and service organization, and the only state geological survey in the nation chartered in a state constitution. The OGS mission focuses on investigating and disseminating information regarding land, water, mineral and energy resources, and promoting sound environmental practices.

Organizationally, the OGS is located within the Mewbourne College of Earth and Energy, and the OGS Director reports to the College Dean. Also located in the College are the ConocoPhillips School of Geology and Geophysics, and the Mewbourne School of Petroleum and Geological Engineering. The ConocoPhillips School of Geology and Geophysics, founded by Charles Gould in 1900, is home to the first school of Petroleum Geology, with the first degree granted in 1904. Charles Gould subsequently became the first Director of the OGS. The Mewbourne School of Petroleum and Geological Engineering is home to the first school of Petroleum Engineering, with the first petroleum engineering degree being granted in 1927.

Candidates should hold a doctorate or have the equivalent experience in geology, geophysics or a closely related field. Prior experience with a public agency, such as the OGS, would be beneficial. If appropriate, the successful candidate may hold a dual appointment as a faculty member within the College. Salary will be commensurate with qualifications and experience.

The Director of the OGS has the responsibility of overseeing activities related to geological and geophysical studies of Oklahoma and adjacent areas, preparation of reports documenting the findings of these studies, communication of these results to individuals and agencies, and engaging the general public as appropriate and/or required.

The position requires supervision and administration of an organization of approximately 40 staff and associated facilities including offices, labs and the Oklahoma Petroleum Information Center (OPIC), which contains an extensive collection of rock cores and samples, other well information and selected facilities for the examination of these cores and samples. It is anticipated that the Director of the OGS will work with Oklahoma universities, state and federal agencies, industry and other entities to conduct research in areas of public interest, as well as provide advice and service in the areas of geology, geophysics and natural resources. The ability to assist OGS personnel in developing programs and proposals to acquire research funding in support of OGS activities will also be a consideration.

The OGS is one of five State Surveys at the University of Oklahoma; the others are the Oklahoma Climatological Survey, Oklahoma Water Survey, Oklahoma Archeological Survey and Oklahoma Biological Survey. Specific activities of the OGS include the following:

- A study of the geological formations of the state with special reference to its natural resources, including coal, oil, gas, asphalt, gypsum, salt, cement, stone, clay, lead, zinc, iron, sand, road building material, water resources and all other mineral resources.
- Management of the Oklahoma seismic recording network, and the reporting and analysis of earthquake activity in the state; an area of current high interest given the recent, significant increase in Oklahoma earthquake activity.
- The preparation and publication of bulletins and reports, accompanied with necessary illustrations and maps, including both general and detailed descriptions of the geological structure and mineral resources of the state.
- The consideration of such other related scientific and economic questions that shall be deemed of value to the people of Oklahoma.

The successful candidate will have the demonstrated experience and ability to oversee these activities, while embracing the public service mission of the OGS and acting as the State Geologist of Oklahoma. Areas of experience that could be considered include an appropriate background with state or national surveys, administration in academia, experience in industry or research, or other related areas.

Review of candidates will begin **October 15th, 2014** and continue until the position is filled. The anticipated starting date is as soon as practical in early 2015. Applicants are requested to submit a complete resume, statement of relevant experience and a list of five references who can be contacted, including names, phone numbers, e-mail addresses and complete mailing addresses. Questions or requests for additional information may be addressed to Larry R. Grillo, Dean of the Mewbourne College of Earth & Energy, and Chair of the OGS Director Search Committee, at (405) 325-3821, or lgrillo@ou.edu. Applications and nominations should be addressed to **OGS Director Search Committee, University of Oklahoma, Sarkeys Energy Center, 100 East Boyd Street, Room 1510, Norman, OK 73019-1008.**

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Multicultural Relationships: Working Across Cultures and Countries

Like travel writers and diplomats, scientists have tremendous opportunities for international interactions. Researchers can train abroad and attend conferences all over the world. The academic sabbatical is a chance to experience a foreign land while maintaining domestic roots. Hosting a guest scientist from another country can bring fresh perspectives to a research group. Cross-border professional relationships require cultivation, though. This feature offers advice from experienced scientists on the whys and hows of international collaborations. By **Chris Tachibana**

Can a single activity revitalize your scientific approach, provide valuable resources for your research, and make a positive contribution to international relations? Scientists often say they receive all these payoffs from global collaborations.

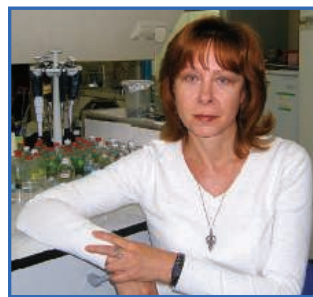
The benefits come with the cost of cultural adjustment, however. Although researchers everywhere share a love of science, different countries have distinct work styles, according to world-traveling scientists. Their descriptions of these styles sound like commentators during an international sports event: Germany is precise, America is confident, Japan is deliberate. Internationally experienced researchers say that overcoming differences in professional norms, expectations, and approaches takes effort, but they overwhelmingly recommend working abroad and hosting international colleagues. Below, six scientists discuss the advantages of global collaborations and offer advice on building productive multicultural relationships.

Three reasons to go global

"The experience of living in a different country and learning different approaches to scientific problems broadens your mind for research," says **Nick Luscombe**, a computational biologist who found that moving from the United Kingdom to the United States for a postdoc was "an eye-opener." The American work culture was "faster, brasher, and more ambitious," he says. "People assumed everything they were working on was a potential *Science* or *Nature* paper." The experience raised his own confidence, but also reinforced his appreciation of time to think through problems. Luscombe now draws on his multicultural experience to lead research groups at University College London, where he will join the new Francis Crick Institute, and the Okinawa Institute of Science and Technology (OIST).

"Nowadays, you have to do complex research to publish," says **Svetlana Dedysh**, head of the Laboratory of Wetland Microbiology, Winogradsky Institute of Microbiology, Russian Academy of Sciences. Dedysh attributes a substantial portion of her professional success to international connections, saying, "My field requires collaboration." Besides microbial ecology, fields that rely on global sharing of samples, data, and methods include climate science, geophysics, and health and science policy. Dedysh was a visiting researcher at Michigan State University in the 1990s and the Max Planck Institute in Marburg, Germany in the 2000s and noticed the detail-oriented and analytic atmosphere in the German laboratories. Like Luscombe, she found the American attitude to be "sparkling enthusiasm, full confidence that everything you are doing is right." She applies both approaches now, for example using enthusiasm to motivate students, although she deploys the American style sparingly, she says, because it takes so much energy. She strongly recommends international collaborations, though. They show people in her group how their work contributes to a broader scientific community.

"Science is a human enterprise that transcends many differences," says **Mónica Feliú-Mójer**, manager of outreach programs for the University of Washington biostatistics department and vice-director of Ciencia Puerto Rico, an organization to advance science in Puerto Rico. Multicultural collaborations unite people from disparate backgrounds and convey positive messages about research, says Feliú-Mójer, including why science should be publicly supported. She encourages her fellow scientists to make **continued>**



Svetlana Dedysh finds the American attitude to be "sparkling enthusiasm, full confidence that everything you are doing is right."

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connections with Hispanic researchers. This promotes science among a growing demographic, she says: “Scientific collaborations can be a bridge to countries in Latin America where we want to have economic and political ties.” Feliú-Mójer went through a professional cultural adaptation herself when she moved from Puerto Rico to Boston after college. In addition to the language and weather, she had to adjust to the scale of U.S. research. “The laboratory where I worked at MIT was the size of the entire department at my university in Puerto Rico,” she says. Researchers with collaborators in countries with limited scientific infrastructure and support, where overnight delivery is a luxury and not standard practice, should be mindful of the bureaucracy and wait times faced by their colleagues, she advises.

Successful global partnerships acknowledge and celebrate cultural differences and anticipate rough spots. A common model says that people encountering a new culture go through highs and lows, with a honeymoon period in which differences are exciting, followed by phases of culture shock and adjustment before mastering the new culture (Black *et al.*, *The Academy of Management Review* **16**, 291 (1991); bit.ly/1t9TRhw). Below, Luscombe, Dedysh, Feliú-Mójer, and other scientists discuss strategies for quickly getting a multinational team to the mastery phrase.

The big barrier: communication

“It’s so easy to feel frustrated by miscommunication,” says Luscombe. “People get personally offended even when they know the problem is just language.” English is the common language of science but the native tongue of only 7% of the world’s population. Non-native speakers often feel that working in a new language flattens their personality and stifles their sense of humor. They can’t make the small talk that builds a relationship. Visiting scientists whose main experience with English has been research articles and other written documents say they struggle with conversations. **Aijie Wang**, distinguished professor and Yangtze River Scholar, Ministry of Education, Harbin Institute of Technology, China, encountered this barrier on a professional development visit to Australia in 2002. “Australians have a strong accent,” she says, “so for the first month I felt like an idiot. I really had to focus, even to understand seminars and workshops.” Attending international meetings and inviting collaborators from other countries is a good way to hone communication skills, she advises, and usually, “it’s not hard to exchange ideas about science.”

Communication across cultures and languages is easier when you’re in the same room, says **Neil Goldsmith**, chief executive officer of Evolva, a biotech company with sites in Switzerland, Denmark, the United States, and India. Evolva was founded by a Brit, a Dane, and a Portuguese, he says, “so we were born multicultural.” A global orientation has clear benefits for a company or research team, says Goldsmith: “People who have lived in more than one country have an openness to new things. And being a small company with a



Aijie Wang says attending international meetings and inviting collaborators from other countries is a good way to hone communication skills and usually, “it’s not hard to exchange ideas about science.”

high level of outside interactions—having a high surface area to volume—makes us learn from other groups and keeps us from talking only to ourselves.” In spite of technology that promises the world from your office, in-person meetings lead to more effective networking and stronger personal connections, says Goldsmith, summarizing, “Trust requires face-to-face.”

Achieving mastery: being a good host

The Luscombe group is the academic version of Evolva, with sites 10,000 km apart in London and Okinawa. Luscombe is the ideal leader for this arrangement. He grew up in Japan, attending an English-speaking school and taking Japanese language classes, an extra task in childhood that, as his parents predicted, he now appreciates. He and his sister went to boarding school in the United Kingdom because their parents wanted them to be comfortable in two cultures.

Both the London and Okinawan groups are a mix of people from multiple countries and Luscombe says that under the right circumstances, this type of group creates its own work culture. Luscombe is committed to teams with a flat structure and well-distributed interactions, so in the larger London group, he tries not to have too many people

of one nationality at once to keep subgroups from forming. For this reason, some multinational laboratories have an English-only policy, so people who share another language don’t start speaking in their common tongue, excluding coworkers.

Luscombe’s group in Okinawa is small enough that no single nationality dominates. However, the team needed time to create a common culture that accommodates different work styles. A simple example, says Luscombe, is that non-Japanese scientists might brainstorm out loud while Japanese scientists prefer thinking through ideas before talking. Whether the differences are cultural or personal, “It takes time to adjust and build trusting, working relationships,” says Luscombe. He maintains a productive research environment by holding videoconferenced meetings in both English and Japanese with the Okinawan group when he is not in Japan. The OIST team also came together around their unique project of studying developmental pathways using marine organisms, says Luscombe. “Now—and I’m not sure [my team will] like this comparison—it’s like a pirate ship. We have people from different exotic backgrounds who left their original countries to be part of this scientific adventure on an island.”

The Center for Microbial Ecology at Michigan State University also has a distinct, global work culture, thanks to director Jim Tiedje, who has hosted more than one hundred international students, postdocs, and visiting scientists. “I don’t think there are any cons,” says Tiedje about hosting guest researchers, “although it’s good to have clear goals.” Find mutually beneficial projects that can be achieved in a realistic timeline, he says. Be clear about expectations and if possible, arrange for multiple visits. Wang visited the Tiedje lab in 2006 and agrees that straightforward discussions at the start of a partnership prevent surprises later. For example, she says, international collaborations taught her **continued>**



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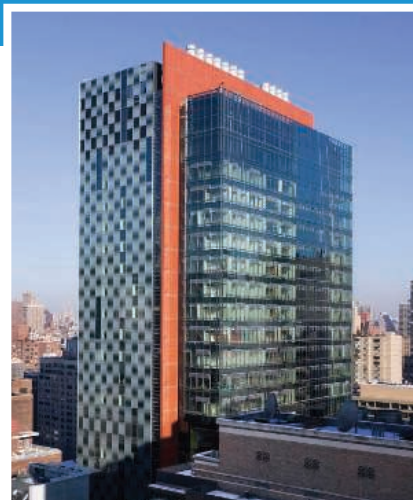
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Communication across cultures and languages is easier when you're in the same room, says Neil Goldsmith.

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the importance of early discussions about publications. "In China," she says, "we expect to honor anyone who helped us by making them coauthors." Working with non-Chinese colleagues, she learned to express clear expectations around authorship from the beginning of a project.

To find international collaborators who will be a good fit, experienced scientists advise looking for people who share your enthusiasm for the field and have innovative ideas. Screen out people who are mainly interested in travel. If possible, follow Goldsmith's principle about face-to-face interactions and meet in person, for example at a conference. At least have an Internet video conversation to test interactions in real-time.

Be sensitive to potential cultural differences when interacting with researchers from another country, says Tiedje, but don't worry too much. "Scientists now have a kind of standard international culture," he says. Dedysh, whose first international research experience was in the Tiedje lab, agrees, saying, "Scientists share an interest in research that is the same all over the world. It helps us recognize each other as colleagues."

When hosting international researchers, look for open-minded visitors who understand that they and their families will change their daily routines, be challenged in simple activities such as shopping, and encounter new and unusual customs. Both Tiedje and Luscombe emphasize the importance of meeting visitors' basic needs. "Get visitors situated with housing and everyday things so those are not a

distraction or worry," says Tiedje. Give new group members a contact person in the lab to answer questions about science and everyday life. Luscombe adds that social support for family members is crucial, saying, "If the family isn't happy, the scientist won't be happy." Recalling his adjustment to life in the United States, Luscombe is empathetic: "I want everyone to feel comfortable in their new country," he says. "I know that for about a year, they will feel out of place and I try to understand if that's reflected in their work. It takes time to settle in." Active participation helps visitors feel at home. At the annual Tiedje holiday dinner, new lab members, including non-Americans, get a full immersion experience: They cook the turkeys.

Achieving mastery: being a good guest

Researchers working with collaborators from different backgrounds might be nervous about making a cultural gaffe or saying something unintentionally offensive. Don't let that hold you back from the tremendous opportunity of making cross-cultural connections, says Feliú-Mójer. If you are unsure about what is culturally appropriate, she says, "Just ask." Particularly if your host seems receptive, your genuine curiosity can spark fun and mutually informative conversations. For example, Feliú-Mójer understands that many people don't know how to use terms like Hispanic, Latino, and Latina. She is an U.S. citizen but identifies first as Puerto Rican, then as Latina, meaning someone from Latin America. She doesn't mind being called Hispanic, indicating a Spanish-speaking person, but understands that people assign different meanings to this term. If it all seems complicated, says Feliú-Mójer, relax, take cues from your hosts, and delight in new customs. And don't be surprised if your Latin American colleague greets you with a kiss.

From her international visits, Dedysh offers two pieces of advice to visiting scientists. At the outset, she says, think about what you can contribute to the collaboration, even if you come from a laboratory with fewer resources. Then, says Dedysh, "be a good, welcome guest." Contribute to the group, but not necessarily as an expert. In fact, Dedysh advises humility, even as a senior scientist working with students. "Don't criticize the lab," she says, "and don't behave as if you are the boss. That will never be helpful." Instead, help out, clean up messes, and be a good lab citizen. Share your expertise if asked and you'll be rewarded with coworkers and friends who want to help you succeed.

To smooth over the inevitable miscommunications, acknowledge and appreciate the extra effort everyone is making. And go in with the right attitude. For positive collaborations across languages and cultures, Goldsmith endorses a principle attributed to Yang Yuanqing, chief executive officer of the computer company Lenovo: "In all situations, assume good intentions."

Chris Tachibana is a science writer based in Seattle, USA, and Copenhagen, Denmark.

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The Purdue University Department of Biological Sciences in conjunction with the Center for Cancer Research and the Walther Cancer Foundation are seeking an outstanding scientist with a proven track record of excellence in the science of cancer structural biology to join our faculty as a Walther Professor in Structural Biology. A Walther Professor is expected to conduct research in the area of structural biology to address fundamental questions in cancer biology; teach undergraduate and/or graduate students, and participate in ongoing programs in the Department of Biological Sciences and the Center for Cancer Research. Preference will be given to candidates utilizing modern cryo-EM approaches combined with other structural approaches such as X-ray crystallography to determine structures of cancer-relevant macromolecules and macromolecular complexes, and/or X-ray crystallography of cancer-relevant drug targets as part of a structure-based drug design program. Candidates should hold the rank of associate or full professor and have a PhD in Biological Sciences or related field, have an excellent track record of publications and extramural funding and a strong commitment to excellence in teaching.

The Department of Biological Sciences offers a dynamic research environment in structural biology research and education. The Markey Center for Structural Biology at Purdue is recognized worldwide for its leadership in structural biology of viruses, membrane proteins, receptors, signaling proteins, enzymes and nucleic acids in addition to methods development in X-ray crystallography, cryo-electron microscopy and NMR. The Purdue Center for Cancer Research is among an elite group of NCI-designated Cancer Centers nationwide and one of only seven centers focused exclusively on basic and translational research. The Walther Professor will have laboratory space in the newly constructed Hockmeyer Hall of Structural Biology, which houses a Titan Krios cryo-TEM, X-ray generators and detectors, and crystallization and imaging robots. Other state-of-the-art shared resources across Purdue such as a Bruker Avance-III 800 MHz NMR and other advanced biophysical instrumentation are available through the Bindley Biosciences and Birk Nanotechnology Centers.

Applications must be submitted electronically to <http://hiring.science.purdue.edu> as a PDF file that includes: a detailed curriculum vitae, names and addresses of three referees, a 2 - 3 page summary of research interests, and a one-page statement of teaching experience and interests. Inquiries should be directed to search@bio.purdue.edu or **Structural Biology Search Committee, Department of Biological Sciences, Purdue University, 915 W. State St., West Lafayette, IN 47907-2054**. Confidential review of applications will begin **October 1, 2014** and will continue until the position is filled. Further information about the Department is available at <http://www.bio.purdue.edu/>.

A background check will be required for employment in this position. Purdue University is an ADVANCE institution and a dual career friendly employer.

Purdue University is EEO/AA Employer. All individuals, including minorities, women, individuals with disabilities, and protected veterans are encouraged to apply.

Florida State University



Coastal & Marine Initiative: Conservation Biology, Fisheries Biology, Population Biology, Community Ecology and Organismal Biology

Florida State University is continuing its *major interdisciplinary initiative in the broadly defined area of Coastal & Marine Research*. During the 2014-15 academic year, the initiative will be recruiting up to five tenure-track faculty members and the search is open with respect to rank. We invite applications in five areas of research of importance to marine and terrestrial coastal areas: (1) conservation biology, (2) fisheries biology, (3) population biology (including demography and population genetics), (4) community ecology (including species interactions and macroecology) and (5) organismal biology (including environmental physiology and functional morphology). We encourage applications from ecologists and evolutionary biologists, empiricists and theoreticians. Habitats of interest include marine habitats (e.g., sea grass, oyster reef, saltmarsh, reefs, open water) and terrestrial systems (e.g., dunes, rivers and streams, maritime forests). Faculty appointments will be in the Department of Biological Science or the Department of Earth, Ocean and Atmospheric Science and can be based at the FSU Coastal and Marine Laboratory. Successful candidates are expected to have a synergistic impact on existing coastal and marine research programs at the University and to contribute to teaching and mentoring at the undergraduate and graduate levels. Successful candidates will be offered highly competitive salaries and start-up packages, high quality research space and access to state-of-the-art instrumentation, computing and facilities in academic and interdisciplinary units.

Applicants are asked to provide a single document in PDF format containing a letter of application, a curriculum vitae, a two page narrative describing their research interests and plans, and a brief teaching statement. Applications must be sent electronically to **coastal-marine2014L.search@fsu.edu**. Applicants should also have three letters of recommendation sent to **coastal-marine2014L.letters@fsu.edu**. The closing date for applications is **November 12, 2014**.

Florida State University is committed to the diversity of its faculty, staff, and students, and to sustaining a work and learning environment that is inclusive. Women, minorities, and people with disabilities are encouraged to apply. FSU is an Equal Opportunity/Access/Affirmative Action Employer.

Florida State University



Coastal & Marine Initiative: Geomorphology, Hydrology, Physical Oceanography and Air-Sea Interactions

Florida State University is continuing its *major interdisciplinary initiative in the broadly defined area of Coastal & Marine Research*. During the 2014-15 academic year, the initiative will be recruiting up to five tenure-track faculty members and the search is open with respect to rank. We invite applications from researchers active in coastal system research in the areas of geomorphology, hydrology, physical oceanography and air-sea interactions. Candidates with research interests that bridge across disciplines, including life sciences, are encouraged to apply. The search seeks to complement the existing strengths in coastal and marine research in the Department of Earth, Ocean and Atmospheric Science, the Department of Biological Science and the FSU Coastal and Marine Laboratory. Faculty appointments will be in the Department of Earth, Ocean and Atmospheric Science and can be based at the Coastal and Marine Laboratory. Successful candidates are expected to have a synergistic impact on existing coastal and marine research programs at the University and to contribute to teaching and mentoring at the undergraduate and graduate levels. Successful candidates will be offered highly competitive salaries and start-up packages, high quality research space and access to state-of-the-art instrumentation, computing and facilities in academic and interdisciplinary units.

Applicants are asked to provide a single document in PDF format containing a letter of application, a curriculum vitae, a two page narrative describing their research interests and plans, and a brief teaching statement. Applications must be sent electronically to **coastal-marine2014P.search@fsu.edu**. Applicants should also have three letters of recommendation sent to **coastal-marine2014P.letters@fsu.edu**. The closing date for applications is **November 12, 2014**.

Florida State University is committed to the diversity of its faculty, staff, and students, and to sustaining a work and learning environment that is inclusive. Women, minorities, and people with disabilities are encouraged to apply. FSU is an Equal Opportunity/Access/Affirmative Action Employer.

The **CUNY Advanced Science Research Center (ASRC)** is devoted to some of global science's most dynamic disciplines – Nanoscience, Photonics, Structural Biology, Neuroscience, and Environmental Sciences – in a highly collaborative research environment. The ASRC operates as a nucleus of a University-wide science enterprise, fostering the development of an integrated research network that brings together faculty, post-doctoral fellows, and students from CUNY's colleges. This state-of-the-art \$350 million center positions CUNY, which is the nation's largest urban public university, at the vanguard of 21st Century scientific exploration and education.

Director of Neuroscience

The ASRC seeks a dynamic and innovative scientist with demonstrated leadership and research accomplishments in neuroscience to serve as Professor and Director of Neuroscience. The position will be a tenured faculty member and program administrator. The successful candidate will be expected to engage in teaching and research; oversee the Neuroscience program; lead researchers in collaborative projects and activities; lead the continued acquisition of external funding; recruit new faculty; and ensure compliance with federal research guidelines and University policies. Applicants must be accomplished and respected researchers in an area of neuroscience with a solid record of scholarly activities and possess appropriate credentials for a senior faculty appointment at one of the CUNY colleges. Successful candidates will be scientists of outstanding merit and accomplishment with active, funded research programs in neuroscience or a closely related area; strong leadership qualities; familiarity with multi-disciplinary programs; interest in promoting research collaborations and diverse academic activities; ability to foster collaboration among scientists; and ability to identify promising new areas for basic research applications. The director will have the opportunity to recruit new faculty into related areas in neuroscience. Exceptional candidates may be appointed as a Distinguished and/or Einstein Professor. Ph.D. in a life science, engineering, or closely-related science area required.

Director of Photonics

The ASRC seeks a dynamic and innovative scientist with demonstrated leadership and research accomplishments in photonics to serve as Professor and Director of Photonics. The position will be a tenured faculty member and program administrator. The successful candidate will be expected to engage in teaching and research; oversee the Photonics program; lead researchers in collaborative projects and activities; lead the continued acquisition of external funding; recruit new faculty; and ensure compliance with federal research guidelines and University policies. Applicants must be accomplished and respected researchers in a Photonics area with a solid record of scholarly activities and possess appropriate credentials for a senior faculty appointment at one of the CUNY colleges. Preference will be given to those whose experimental research focus areas include but are not limited to one or more of the following: nanophotonics, terahertz technology, ultrafast spectroscopy, and plasmonics. Successful candidates will be scientists of outstanding merit and accomplishment with active, funded research programs in Photonics or a closely related area; strong leadership qualities; familiarity with multi-disciplinary programs; interest in promoting research collaborations and diverse academic activities; ability to foster collaboration among scientists; and ability to identify promising new areas for basic research applications. The director will have the opportunity to recruit new faculty into related areas in photonics. Exceptional candidates may be appointed as a Distinguished and/or Einstein Professor. Ph.D. in a life science, engineering, closely-related science discipline, or Photonics specialty area required.

Faculty Positions in Nanoscience, Structural Biology and Environmental Sciences

The ASRC seeks to appoint a number of outstanding, ambitious, and highly innovative scientists with demonstrated world-class research accomplishments to serve as faculty in the areas of **Nanoscience, Structural Biology, and Environmental Sciences**. Faculty are expected to make key contributions in establishing the ASRC as an internationally leading research center, through innovative and collaborative research for societal and economic benefit, and by inspiring new generations of scientists.

To apply and to seek further information visit:
asrc.cuny.edu/jobs

**CUNY Advanced Science Research Center
85 St. Nicholas Terrace, New York, NY 10031**

We are committed to enhancing our diverse academic community by actively encouraging people with disabilities, minorities, veterans, and women to apply. We take pride in our pluralistic community and continue to seek excellence through diversity and inclusion. EO/AA Employer.



Faculty Positions – Cancer Systems Biology

Rutgers Cancer Institute of New Jersey (CINJ) invites applications for tenure-track faculty positions specializing in cancer systems biology. Exceptional senior investigators will also be considered. CINJ is an NCI-designated, comprehensive and consortium cancer center that includes faculty members from Princeton University.

New faculty are expected to establish an innovative, independent and collaborative research program addressing important, fundamental questions in the area of cancer systems biology. Interdisciplinary approaches are encouraged and the combined CINJ consortium faculty has complementary expertise and strength in cancer biology, signal transduction, systems biology, metabolism, and genomic instability.

Exceptional opportunities exist at the cancer center for collaborations with strong research programs, including clinical trials research, cancer chemoprevention, clinical and basic pharmacology, Phase 1 clinical trials, as well as abundant opportunities for clinical and scientific interactions with internationally known investigators in the basic science and clinical departments. Qualified candidates must have a PhD. Rank will be commensurate with experience.

Rutgers Cancer Institute of New Jersey (CINJ) is one of only 41 NCI-designated Comprehensive Cancer Centers, the only such center in New Jersey, and a leader in laboratory, clinical prevention and public health science. CINJ offers world-class quality cancer care providing the most advanced medicines and treatment options for patients in its New Brunswick facility, as well as at its network of sixteen hospitals across the state. CINJ manages more than 90,000 patient visits per year and the CINJ network of hospitals provides care for approximately 35 to 40 percent of all cancer patients in the state of New Jersey.

Interested applicants should send their CV, a brief description of their research interests and plans, and the names of three references via email to CINJobs@gmail.com addressed to: David J. Foran, PhD, Professor Pathology, Laboratory Medicine & Radiology, CIO and Director of Biomedical Informatics, Rutgers Cancer Institute of New Jersey, c/o Larissa Varela, Faculty Recruitment Coordinator.

Rutgers, the State University of New Jersey, is an Equal Opportunity/Affirmative Action employer, and is compliant with the Americans with Disabilities Act (ADA). Women and minorities are encouraged to apply. For more information, please visit <http://jobs.rutgers.edu/TheRUCCommitment.htm>.



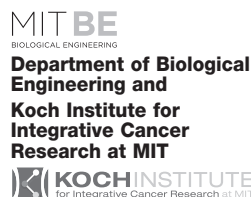
The Center for Regenerative Medicine (CRM) and the Center for Vital Organ Engineering at Massachusetts General Hospital along with the Harvard Stem Cell Institute invite applications for **two** tenure-track Assistant Professor positions. Outstanding scientists in the field of vertebrate stem cell or regenerative biology will be considered. Candidates with a research program focused on tissue regeneration, plasticity and function and who have a demonstrated interest in application to human disease are especially welcome. Successful candidate(s) will be members of the **Harvard Stem Cell Institute** and faculty of **Harvard Medical School**. Candidates must hold a PhD and/or MD and have a history of innovative, interactive research.

Applicants should send an electronic copy of a (1) letter of interest, (2) research plan, (3) current curriculum vitae and (4) three letters of recommendation to **Dr. David Scadden c/o Miklosik.Mona@mgh.harvard.edu, Center for Regenerative Medicine Search Committee, Massachusetts General Hospital, 185 Cambridge St., CPZN 4258, Boston, MA 02114.**

Women and minority candidates are urged to apply. MGH is an Equal Opportunity/Affirmative Action Employer.



Massachusetts Institute of Technology



Faculty Position

The Biological Engineering Department (<http://web.mit.edu/be>) and Koch Institute for Integrative Cancer Research (<http://ki.mit.edu/>) at the Massachusetts Institute of Technology invite applications for an assistant or untenured faculty appointment. In special cases, a senior faculty appointment may be possible.

The mission of the Department of Biological Engineering is to define and establish a new discipline fusing molecular life sciences with engineering, to advance fundamental understanding of how biological systems operate and to develop effective biology-based technologies. The Koch Institute is an NCI-designated Cancer Center which features research across a wide range of areas in cancer biology and cancer-oriented engineering. This is an open search with regard to field of study and specific research focus, but clear cancer relevance is essential.

Applicants should hold a PhD in a science or engineering discipline related to biological engineering. Areas of interest include, but are not limited to: imaging, computational modeling, systems biology, biomolecular design, and novel approaches to detecting, monitoring and treating cancer. The candidate will be expected to develop and lead an internationally competitive research program. Faculty duties include teaching at the undergraduate and graduate levels, advising students, conducting original scholarly research and developing course materials at the graduate and undergraduate levels.

Applicants should hold a PhD in a science or engineering discipline related to biological engineering by the start of employment. The successful candidate will have laboratory space in the Koch Institute.

Applicants should include curriculum vitae, brief summaries of past accomplishments and a description of future research plans. Letters of recommendation should be sent separately from three scientists/engineers that evaluate the candidate's accomplishments and future potential for both research and teaching.

Responses by November 15, 2014, will be given priority. Questions may be directed to Prof. Douglas Lauffenburger, MIT Biological Engineering Department Head; lauffen@mit.edu. Please upload all application materials online at: <https://school-of-engineering-faculty-search.mit.edu/>

We especially encourage minorities and women to apply because of MIT's strong commitment to diversity in education, research and practice. MIT is an Equal Opportunity/Affirmative Action employer.

<http://web.mit.edu>

Assistant or Associate Professor



The Department of Cancer Biology at the Perelman School of Medicine at the University of Pennsylvania seeks candidates for a Full, Assistant, and/or Associate Professor position in the tenure track. Rank will be commensurate with experience. The successful applicant will have experience in the field of cancer biology, including but not limited to cancer genetics and genomics, tumor microenvironment, cancer cell metabolism and stem cells. Responsibilities include the development of an independent research program. Teaching duties comprise mentoring students and course lecturing. Applicants must have an M.D. and/or Ph.D degree and have demonstrated excellent qualifications in research and education.

We seek candidates who embrace and reflect diversity in the broadest sense.

The University of Pennsylvania is an EOE. Minorities/Women/Individuals with disabilities/Protected Veterans are encouraged to apply.

Apply for this position online at: https://www.med.upenn.edu/apps/faculty_ad/index.php/g/d3721



Faculty Positions Open

Marine Science
Medical Physics
Biology

Okinawa Institute of Science and Technology (OIST) is a new, English-language graduate university offering a world-class research environment and a highly diverse, international research community with faculty, staff and students from over 40 countries. It is located in Okinawa on a beautiful campus overlooking the East China Sea.

Suitably qualified candidates will be appointed as Professors, Associate Professors or Assistant Professors. Professors and Associate Professors are usually appointed with tenure; Assistant Professors are tenure-track appointments, with an initial period of 7 years and a tenure review in the 5th year. The scope of this broad search is guided by the following:

- ◆ *At least one tenure-track professor in **Marine Science**, including physiology, marine genomics and coastal ecology (Reference V1401)*
- ◆ *Two appointments, at least one of which is a tenured professor, specializing in the **Medical Physics** area covered by Radiation Physics, Radiobiology and Medical Imaging (Reference V1402, V1403)*
- ◆ *At least one appointment, to include a tenured professor, in **Biology**, including cell biology, mammalian developmental biology and stem cell biology (Reference V1404)*

For all positions we seek applicants with strong interdisciplinary interests and outstanding scientific records of creativity and productivity. Detailed descriptions of all posts and the application procedure may be accessed at <https://groups.oist.jp/facultypositions>

Inquiries about any of these positions should be directed to Professor Ken Peach, Dean for Faculty Affairs, OIST, faculty-recruiting@oist.jp

Applications should be made online before 30th November 2014.

OIST Graduate University is an equal opportunity, affirmative action educator and employer and is committed to increasing the diversity of its faculty. We strongly encourage women and minority candidates to apply.



Call for Assistant Professors and Professors



IST Austria invites applications for **Tenure-Track Assistant Professor** and **Tenured Professor** positions to lead independent research groups in all areas, as well as cross-disciplinary areas of

- **BIOLOGY:** Applicants using quantitative approaches at the interface between experiments and theory are particularly encouraged to apply.
- **CHEMISTRY:** Applicants at the interface to Physics or Biology are particularly encouraged to apply. While at present our main focus is on experimental chemistry, outstanding theoreticians will be considered as well.
- **NEUROSCIENCE** (such as molecular, cellular, systems neuroscience): Applicants using advanced imaging and/or cutting edge molecular techniques are particularly encouraged to apply.
- **PHYSICS:** Applicants in Atomic, Molecular, and Optical Physics, Condensed Matter Physics, Bio- and Soft Matter Physics are particularly encouraged to apply. While at present our main focus is on experimental physics, outstanding theoreticians will be considered as well.

IST Austria is a recently founded public institution dedicated to basic research and graduate education near Vienna. Currently active fields of research include biology, neuroscience, physics, mathematics, and computer science. IST Austria is committed to become a world-class research center with up to 1000 scientists and doctoral students by 2026. The institute has an interdisciplinary campus, an international faculty and student body, as well as state-of-the-art-facilities. The working language is English.

Successful candidates will be offered highly competitive research budgets and salaries. Faculty members are expected to apply for external research funds and participate in graduate teaching. Candidates for senior positions must be internationally accomplished scientists in their respective fields.

DEADLINES: Open call for Professor applications. For full consideration, Assistant Professor applications should arrive on or before November 15, 2014. Application material must be submitted online: www.ist.ac.at/professor-applications

IST Austria values diversity and is committed to equal opportunity. Female researchers are especially encouraged to apply.



Fundamental Neuroscience Faculty

Florida State University



Brain Health & Disease Initiative: Fundamental Neuroscience

As part of a strategic campus-wide investment in neuroscience, Florida State University invites applications for up to five tenure-track positions. This faculty search is open with respect to rank and academic department, including departments represented in the interdisciplinary Program in Neuroscience (www.neuro.fsu.edu). Areas of interest span molecular, genetic, cellular and physiological mechanisms underlying normal and disordered brain function. Areas of research specialization include, but are not limited to: (1) In vivo circuit function including transgenic, optogenetic, electrophysiological, molecular genetic, developmental and/or computational analyses; (2) Mechanisms of sensory function, learning and memory, circadian rhythms, neural regulation of metabolism, and motivated behavior, including drug addictions, social and ingestive behaviors; and (3) Mechanisms of neurodegenerative disease, disorders of mood and emotion, functional alterations in aging and prospects for brain repair. Successful candidates are expected to have a synergistic impact on existing research programs contribute to teaching and mentoring students. Successful candidates will be offered highly competitive salaries and start-up packages, research space in modern laboratory buildings and access to state of the art core facilities.

Applicants are asked to provide a *single document* in PDF format containing a letter of application, a full CV that includes links to recent publications, a two page narrative describing their research interests, and a brief teaching statement to brain-initiative2014N.search@fsu.edu. Applicants should also have three letters of recommendation sent in electronic format to brain-initiative2014N.letters@fsu.edu. The search committee will begin reviewing applicants starting **November 1, 2014** and will continue to review new applications until the position is filled.

The Florida State University is committed to the diversity of its faculty, staff, and students, and to sustaining a work and learning environment that is inclusive. Women, minorities, and people with disabilities are encouraged to apply. FSU is an Equal Opportunity/Access/Affirmative Action Employer. As an agency of the State of Florida, all application materials and selection procedures are available for public review.

Florida State University

Brain Health & Disease Initiative: Translational Health and Human Neuroscience



As part of a strategic campus-wide investment in neuroscience, including human neuroimaging, Florida State University invites applications for up to five tenure-track positions. This faculty search is open with respect to rank and academic department. Areas of interest include but are not limited to research of normal and abnormal behavioral and cognitive functions across the life span and brain mechanisms underlying medical, psychiatric, neurodevelopmental, and neurodegenerative disorders. We are particularly interested in candidates who use innovative methodologies and approaches to the study of brain function including fMRI and other imaging methods. Successful candidates are expected to have a synergistic impact on existing research programs and evidence of the ability to attract external research support. Highly competitive salaries and start-up packages, state-of-the-art research space and access to world-class instrumentation and facilities in academic and interdisciplinary units will be offered to successful candidates.

Applicants are asked to provide a *single document* in PDF format containing a letter of application, a full CV (with links to recent publications), a two page narrative describing their research interests, and a brief teaching statement. Applications must be sent electronically to brain-initiative2014H.search@fsu.edu. Applicants should also arrange to have three letters of recommendation sent in electronic format to brain-initiative2014H.letters@fsu.edu. The search committee will begin reviewing applications starting **November 1, 2014** and will continue to review new applications until the position is filled.

The Florida State University is committed to the diversity of its faculty, staff, and students, and to sustaining a work and learning environment that is inclusive. Women, minorities, and people with disabilities are encouraged to apply. FSU is an Equal Opportunity/Access/Affirmative Action Employer. As an agency of the State of Florida, all application materials and selection procedures are available for public review.

UNIVERSITY of CALIFORNIA Office of the President

UC OBSERVATORIES DIRECTOR

The University of California (UC) seeks a new Director of the University of California Observatories (UCO). UCO is a multi-campus research unit responsible for the suite of world-class optical and infrared observatories shared by astronomers across the UC system. These facilities include Lick Observatory, Keck Observatory, and the upcoming Thirty Meter Telescope Observatory. UCO is headquartered at UC Santa Cruz, with instrumentation labs at UC Santa Cruz and UCLA and participation from nine UC campuses and two national labs.

The successful candidate will have an outstanding research record in astronomy and astrophysics, vision and demonstrated leadership, knowledge of astronomical instrumentation, and experience in leading and administering large astronomical projects or organizations.

Applications should include a summary of scientific accomplishments, management, and administrative experience, CV, and three professional references. Send applications to UCODirectorSearch@ucop.edu. Apply by October 31, 2014 for full consideration.

Further info: <http://ucal.us/ucodirectorsearch>

University of California is an
Equal Opportunity/Affirmative Action employer.



Postdoctoral Fellows

Postdoctoral Fellow to participate in research related to cardiovascular disease and diabetes. The candidate must have a Ph.D. in Biomedical Sciences or in Chemical Sciences with a strong background in analytical chemistry. Advanced expertise in mass spectrometry, gas chromatography, HPLC etc. is desirable.

Postdoctoral Fellow to study aldehyde metabolism in cardiovascular disease is required. The candidate is expected to have a PhD degree with expertise in biochemical and molecular biological techniques. Familiarity with fatty acid metabolism, oxidative stress, aldehyde metabolizing enzymes, and chemical reactions preferred. To apply, submit a CV, a brief statement of your qualifications, research interest and future career plans as well as names and contact information of three references to spartha@ucf.edu

UCF is an equal opportunity/affirmative action employer and encourages applicants from minorities, women, and other underrepresented groups. Search materials are available for public review as provided by Florida statute.



UNIVERSITY OF CENTRAL FLORIDA
College of Medicine



ÉCOLE POLYTECHNIQUE
FÉDÉRALE DE LAUSANNE

Faculty Position in Biomaterials at the Ecole polytechnique fédérale de Lausanne (EPFL)

The School of Engineering of EPFL invites applications for a **tenure track assistant professor in biomaterials** within its Institute of Materials with a possible joint appointment in the Institute of Bioengineering. We seek exceptional individuals who will develop and drive a research program at the forefront of the discipline, who have a strong dedication to teaching at the undergraduate and graduate levels, and who will be proactive members of a vibrant Materials community.

Top-level applications covering all areas of biomaterials science and engineering are invited including, but not limited to biomolecular, biomimetic, bio-inspired and biomedical materials.

Start-up resources and state-of-the-art research infrastructure will be available. Salaries and benefits are internationally competitive.

The Institute of Materials at EPFL is well integrated in the School of Engineering and has close interactions with the Institute of Bioengineering. Further exciting opportunities for interactions exist with the Schools of Life Sciences and Basic Sciences, as well as with the newly founded Wyss Center and the University Hospital of Lausanne (CHUV).

EPFL, with its main campus located in Lausanne, Switzerland, is a dynamically growing and well-funded institution fostering excellence

and diversity. It has a highly international campus at an exceptionally attractive location boasting first-class infrastructure. As a technical university covering essentially the entire palette of engineering and science, EPFL offers a fertile environment for research cooperation between different disciplines. The EPFL environment is multi-lingual and multi-cultural, with English often serving as a common interface.

Applications should include a cover letter with a statement of motivation, curriculum vitae, list of publications and patents, concise statement of research and teaching interests, and the names and addresses of at least five referees. Applications must be uploaded in PDF format to the recruitment web site: <http://go.epfl.ch/imx-search>

Formal evaluation of candidates will begin on **December 1st, 2014**.

Enquiries may be addressed to:

Prof. Harm-Anton Klok
Search Committee Chair
e-mail: imx-search@epfl.ch

For additional information on EPFL, please consult the web sites: www.epfl.ch, sti.epfl.ch, imx.epfl.ch and ibi.epfl.ch.

EPFL is committed to increasing the diversity of its faculty, and strongly encourages women to apply.



ÉCOLE POLYTECHNIQUE
FÉDÉRALE DE LAUSANNE

Faculty Position in Materials Electron Microscopy at the Ecole polytechnique fédérale de Lausanne (EPFL)

The School of Engineering of EPFL invites applications for a **tenure track assistant professor in electron microscopy of materials** within its Institute of Materials. We seek exceptional individuals who will develop and drive a research program at the forefront of the discipline, who have a strong dedication to teaching at the undergraduate and graduate levels, and who will be proactive members of a vibrant Materials community.

Top-level applications are invited from candidates at the cutting edge of electron microscopic imaging as applied to materials science and engineering. Areas of interest include, but are not limited to, high-resolution imaging, 3D and 4D imaging, advanced analysis methods, and innovative modeling approaches, as applied to the structural characterization of materials by means of electron microscopy.

Start-up resources and the state-of-the-art research infrastructure at EPFL's Interfaculty Center for Electron Microscopy (CIME) will be made available for the successful candidate. Salaries and benefits are internationally competitive.

EPFL, with its main campus located in Lausanne, Switzerland, is a dynamically growing and well-funded institution fostering excellence and diversity. It has a highly international campus at an exceptionally attractive location boasting first-class infrastructure. As a technical university

covering essentially the entire palette of engineering and science, EPFL offers a fertile environment for research cooperation between different disciplines. The EPFL environment is multi-lingual and multi-cultural, with English often serving as a common interface.

Applications should include a cover letter with a statement of motivation, curriculum vitae, list of publications and patents, concise statement of research and teaching interests, and the names and addresses of at least five referees. Applications must be uploaded in PDF format to the recruitment web site: <http://go.epfl.ch/emm-search>

Formal evaluation of candidates will begin on **December 1st, 2014**.

Enquiries may be addressed to:

Prof. Harm-Anton Klok
Search Committee Chair
e-mail: emm-search@epfl.ch

For additional information on EPFL, please consult the web sites: www.epfl.ch, sti.epfl.ch, imx.epfl.ch and cime.epfl.ch.

EPFL is committed to increasing the diversity of its faculty, and strongly encourages women to apply.



Jefferson Science Fellowship



The National Academies is pleased to announce a call for nominations and applications for the 2015 Jefferson Science Fellows program. Initiated by the Secretary of State in 2003, this fellowship program engages the American academic science, technology, engineering and medical communities in the design and implementation of U.S. foreign policy.

Jefferson Science Fellows (JSF) spend one year at the U.S. Department of State or the U.S. Agency for International Development (USAID) for an on-site assignment in Washington, D.C. that may also involve extended stays at U.S. foreign embassies and/or missions.

The fellowship is open to tenured, or similarly ranked, academic scientists, engineers and physicians from U.S. institutions of higher learning. Nominees/applicants must hold U.S. citizenship and will be required to obtain a security clearance.

The deadline for 2015-2016 program year applications/nominations is **January 12, 2015**. To learn more about the Jefferson Science Fellowship and to apply, visit the website at:

www.national-academies.org/jsf

THE NATIONAL ACADEMIES
Advisers to the Nation on Science, Engineering, and Medicine



**Massachusetts
Institute of
Technology**

Come work with us!

Tenure Track Faculty

The Department of Brain & Cognitive Sciences (BCS) (<http://bcs.mit.edu>) and The Picower Institute for Learning & Memory at MIT (<http://picower.mit.edu>) are looking to hire up to three (3) tenure-track faculty at the assistant professor level who work in one or more of the following three (3) areas:

- i) *Computational* approaches to intelligence, cognition or neuroscience; an experimental component to the candidate's research would be viewed as a positive but is not necessary. An affiliation with Electrical Engineering and Computer Science, the Computer Science and Artificial Intelligence Laboratory (CSAIL), or other allied departments is possible.
- ii) *Molecular & cellular*: The Picower Institute is searching for a candidate studying development, function or plasticity of neuronal circuits at the cellular, circuit, and/or systems levels using a multi-faceted approach combining different methodologies and levels of analysis. Candidates with strong cellular/molecular training who are studying development of brain circuits or using stem cell technologies are particularly encouraged to apply.
- iii) *Human cognition and/or cognitive neuroscience* using behavioral methods, especially in the areas of language and/or cognitive development OR using fMRI/neuroscience methods.

Successful applicants are expected to develop and lead independent, internationally competitive research programs and to share in our commitment to excellence in undergraduate and graduate education by teaching courses and mentoring graduate and undergraduate research. PhD must be completed by start day of employment and some postdoctoral training is preferred.

Please submit application materials – cover letter, CV, statement of research and teaching interests and representative reprints – online at <https://academicjobsonline.org/ajob/jobs/4202>. Please state research area in cover letter. To help direct the application, applicants should indicate which of the three areas listed above is their main research area by answering the mandatory questions included in the application. In addition, please arrange to have three letters of recommendation submitted online. Review of applications will begin on November 1, 2014.

MIT is an affirmative action employer, and we encourage applications from women and underrepresented minorities.

<http://web.mit.edu>



BioFrontiers Institute
UNIVERSITY OF COLORADO

Tenure-Track Assistant Professor in Computational Biology at the University of Colorado BioFrontiers Institute

BioFrontiers integrates faculty from nine departments to address significant problems in biology and medicine at the interface of the biological sciences with the physical sciences, mathematics, computer science, and/or engineering (see <http://BioFrontiers.colorado.edu/about>). While a successful candidate could be rostered in any of our participating departments, this search will focus on candidates seeking to develop an internationally recognized research program in computational biology, with additional emphases on genomics, the microbiome, and/or network science. Successful candidates must demonstrate a strong focus on innovative computation in their research plan.

The tenure-track position is at the Assistant Professor level, although more senior candidates will also be considered. Candidates must have a Ph.D. and a demonstrated commitment to teaching at undergraduate and graduate levels. The successful candidate will hold the Marvin H. Caruthers Endowed Chair for Early Career Faculty for a period of four years, after which the Chair will be recycled to another early career faculty member.

Application materials are accepted electronically at <http://www.jobsatcu.com/postings/86204>. Review of applications will begin on **November 11, 2014** and will continue until the position is filled. The University of Colorado Boulder conducts background checks for all final applicants.

As an Equal Opportunity/Affirmative Action Employer, the University of Colorado is committed to diversity and equality in education and employment and sensitive to the needs of dual-career couples.

ASSISTANT PROFESSOR Environmental Policy ARTS AND SCIENCE

Qualified applicants are invited to apply for a full-time, tenure-track assistant professor position in the Department of Environmental Studies, to begin September 1, 2015, pending administrative and budgetary approval. We seek an excellent scholar with a rigorous theoretical and empirical approach to environmental studies. We are especially interested in candidates with environment-expertise in policy, governance, and environmental affairs. Candidates with a Ph.D. in environmental studies or relevant discipline are invited to apply. The successful candidate will be expected to teach and contribute broadly to environmental studies. Candidates should possess a Ph.D. by September 2015, have a research program that demonstrates the potential to be a leader in the field of Environmental Studies, and have proven the ability to be an excellent teacher. The successful candidate will have the opportunity to affiliate with other NYU departments, programs, and centers such as the Wagner School of Public Service at NYU.

*Application deadline is **November 15, 2014**.* To learn more and apply, see the NYU Environmental Policy web site at <http://environment.as.nyu.edu>.



NEW YORK UNIVERSITY

NYU is an Equal Opportunity/Affirmative Action Employer.

Faculty Positions at The Wistar Institute

As the nation's first independent biomedical research Institute with a tradition of landmark discoveries in the areas of cancer, immunology and vaccines, The Wistar Institute has embarked on or upon its largest facility and faculty expansion. Enabled by the recent opening of a new, state-of-the-art Robert and Penny Fox Research Tower, and renewal of its NCI-designated Cancer Center designation with an Exceptional rating, the Institute now seeks outstanding faculty candidates at all academic ranks (Assistant, Associate and Full Professor) for multiple faculty positions in its four Cancer Center Programs: Gene Expression and Regulation, Translational Tumor Immunology, Tumor Microenvironment and Metastasis, and Molecular and Cellular Oncogenesis.

Successful candidates will leverage a highly collaborative and inclusive institutional culture to develop or expand extramurally-funded research programs in basic and translational molecular cancer research. Investigators working in any cancer type are welcomed to apply although ovarian cancer and melanoma are particular focus areas in the Cancer Center. Key areas of programmatic interest include, but are not limited to:

- Cancer epigenetics, biochemistry and molecular biology, functional genomics, gene expression regulation, non-coding RNAs, tumor virology;
- Tumor suppressor and oncogenes, cancer stem cells, cancer metabolomics;
- Tumor immunology;
- Metastasis and tumor microenvironment;
- Systems and computational biology of cancer development and treatment resistance;
- Chemical biology, new target identification and biomarker assessment.

The Wistar Institute offers highly competitive start-up support, salary and fringe benefits in addition to an outstanding and highly interactive research environment. Faculty will be recruited to new research space with a state-of-the-art vivarium and outstanding core facilities in proteomics, genomics, imaging, molecular screening, bioinformatics, flow cytometry, and others. The Institute's location adjacent to the University of Pennsylvania campus provides for vigorous academic and clinical collaborations, as well as attractive training opportunities for graduate students and postdoctoral fellows.

Applications will be reviewed as received and will be accepted until all positions are filled. To ensure timely consideration during the current recruitment cycle, applicants should submit applications before **November 1, 2014**. The application should include: a curriculum vitae, a brief summary of past and future research interests, history of research funding support (if applicable). Applications (submitted as a single PDF) should be sent by e-mail to: **Maria Colelli, Faculty Search Coordinator (colelli@wistar.org), The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104. EOE/AA/M/F/D/V.**



Cleveland Clinic

CHAIR

Department of Molecular Cardiology

LERNER RESEARCH INSTITUTE, CLEVELAND CLINIC

We are seeking a Chair for the Department of Molecular Cardiology which occupies 38,000 sq. ft. in the Lerner Research Institute. An endowed chair accompanies this position. The department currently consists of 15 faculty members (including Ed Plow, Ph.D. who recently decided after 22 years to step down as Chair) with well-funded research programs in cardiovascular biology. The ideal applicant for this position will have an outstanding national reputation in areas that complement the strengths of the department. The Lerner Research Institute with over 160 independent investigators in 11 departments and an annual budget of greater than \$250 million has a commitment of excellence in basic, translational & clinical research. The Chair will be provided with a highly competitive salary, generous start-up support, and recruitment packages for additional faculty.

Curriculum vitae, a personal statement and the names and addresses of three references should be sent to:

Raed A. Dweik, MD, Search Committee for the Chair of Molecular Cardiology

Departments of Pulmonary and Critical Care Medicine / Respiratory Institute and Pathobiology / Lerner Research Institute, Desk A-90,
Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195.

Or apply online at www.clevelandclinic.org/physicianrecruitment

Visit us on the Web at <http://www.lerner.ccf.org/moleccard/>

E-mail: dweikr@ccf.org

Equal Employment/Affirmative Action Employer – Min/Fem/Disability/Vet



**DEVELOPMENTAL BIOLOGIST
ASSISTANT PROFESSOR (TENURE-TRACK)**
DEPARTMENT OF BIOLOGICAL SCIENCES
COLLEGE OF SCIENCE

Responsibilities: This is a tenure-track faculty position and the successful candidate will be expected to establish and maintain a vigorous, extramurally funded research program in the areas of eukaryotic developmental biology at LSU. Biological Sciences is a large and dynamic department, with research ranging across all levels of biological organization from molecules to ecosystems. The successful candidate will complement these strengths and contribute to undergraduate and graduate teaching.

Required Qualifications: Ph.D. in Biological Sciences or related field; successful track record of independent research.

Additional Qualifications: Postdoctoral experience preferred.

An offer of employment is contingent on a satisfactory pre-employment background check. Application deadline is November 30, 2014, or until a candidate is selected. Apply online and view a more detailed ad at: <https://lsusystemcareers.lsu.edu> Position #001309

Quick link at ad URL: <https://lsusystemcareers.lsu.edu/applicants/Central?quickFind=58209>

LSU IS AN EQUAL OPPORTUNITY/EQUAL ACCESS EMPLOYER



**ECOLOGIST
ASSISTANT PROFESSOR (TENURE-TRACK)**
DEPARTMENT OF BIOLOGICAL SCIENCES
COLLEGE OF SCIENCE

Responsibilities: This is a tenure-track faculty position and the successful candidate will be expected to establish a vigorous, extramurally funded research program in Population, Community or Theoretical Ecology at LSU, and contribute to undergraduate and graduate teaching.

Required Qualifications: Ph.D. in a Biological Sciences or related field; successful track record of independent research. A.B.D. candidates will be considered as long as degree is completed by time of appointment.

Additional Qualifications: Postdoctoral experience preferred; broadly trained ecologist who addresses questions regarding population demography and regulation, community structure and species interactions, or the maintenance and function of biodiversity.

An offer of employment is contingent on a satisfactory pre-employment background check. Application deadline is October 31, 2014, or until a candidate is selected. Apply online and view a more detailed ad at: <https://lsusystemcareers.lsu.edu> Position #027662

Quick link at ad URL: <https://lsusystemcareers.lsu.edu/applicants/Central?quickFind=58210>

LSU IS AN EQUAL OPPORTUNITY/EQUAL ACCESS EMPLOYER



Faculty Position
**University of Pittsburgh Cancer Institute and
Department of Pharmacology & Chemical Biology**

Applications are invited for a tenured faculty position at the level of Associate Professor at the University of Pittsburgh Cancer Institute (UPCI). Candidates who have a PhD, MD or equivalent graduate degree are being recruited to expand expertise in cancer biology and treatment. The incumbent will have primary appointment in the Department of Pharmacology & Chemical Biology, University of Pittsburgh School of Medicine (UPSOM).

The UPCI has consistently been among the top NCI-funded and NCI-designated Comprehensive Cancer Centers located within the academic environment of the University of Pittsburgh and University of Pittsburgh Medical Center and has an active and highly interactive community of basic, translational and clinical research scientists. The institution has several state-of-the-art facilities with varied capabilities including cell and tissue imaging, high-throughput drug discovery and a cancer biomarkers facility with mass spectrometry, genomics and proteomics platforms.

In order to develop an outstanding, extramurally-supported research program, the applicants must have excellent communication skills, publications in high-impact journals and a proven track record of extramural funding. The incumbent will have the opportunity to mentor graduate students through the Department of Pharmacology and Chemical Biology and UPSOM Integrated Biomedical Graduate Program and will have progressive involvement in the teaching of graduate and medical students. Salary and benefits will be commensurate with experience.

Interested candidates should send curriculum vitae, brief description of research interests and contact information for three references to: **UPCI Search Committee/Pharmacology, Dr. Maryann Donovan, Associate Director for Research Administration, UPCI, C/O Lola Thompson, 5150 Centre Avenue, Suite 532, Pittsburgh, PA 15232; Email: thompsonla3@upmc.edu.**

*The University of Pittsburgh is an
Affirmative Action, Equal Opportunity Employer.*



Faculty Positions in Basic Translational Sciences

The UNC Lineberger Comprehensive Cancer Center, in collaboration with departments in the School of Medicine and across the entire University of North Carolina Chapel Hill, seeks outstanding candidates for faculty positions at all levels and at all ranks in basic and translational cancer research with a special interest in a senior cancer researcher. This broad-based recruitment seeks outstanding scientists in a number of areas, including but not limited to: animal models, signal transduction, computational and systems biology, cancer genetics, virology, drug development and target validation, epigenetics and gene expression, DNA damage and repair, cancer therapy, cancer immunology, inflammation and cancer, and stem cells. Applicants should have a strong record of recent accomplishments as a post-doctoral fellow or sustained productivity as an established faculty member. Appointment and rank in an academic department will be determined by the applicant's qualifications. Applications will be reviewed beginning December 1, 2014 and until the positions are filled.

Educational Requirements: Doctoral Degree
Qualifications and Experience: Doctoral Degree

Apply online at <http://unc.peopleadmin.com/postings/50871> and provide curriculum vitae, a list of four references, and Research Statement.

The University of North Carolina at Chapel Hill is an Equal Opportunity Employer. Women and minorities are strongly encouraged to apply and self-identify on their application.



Faculty Position in the Life Sciences

The Stowers Institute for Medical Research invites innovative young scientists in the Life Sciences to submit applications for a faculty position. We anticipate making an appointment in 2015 at the rank of Assistant Investigator. Research programs of interest include, but are not limited to: computational biology, neuroscience, developmental and cell biology, genomics, stem cell biology, regenerative biology and epigenetics. Our interests encompass a broad gamut of experimental organisms and approaches. The successful candidate will benefit from and complement the Institution's existing strengths in genetics and epigenetics, cell and chromosome biology, stem cells and regenerative biology, developmental biology and evolution, and biochemistry and neuroscience.

The position is fully funded throughout the candidate's appointment. This includes \$600,000 per year for full salary support and research funding, in addition to start-up funds and ongoing needs for equipment. The initial appointment is for 6 years and is then subject to renewal every 6 to 7 years. In total, the package for a junior position is more than \$3.5 million over the first term and increases significantly after promotion. In addition, investigators may take advantage of exceptional core facilities and technology centers staffed by over 100 scientists. Stowers investigators have multiple opportunities to be involved in the Institute's Graduate School program.

Candidates must have a Ph.D. or equivalent degree and postdoctoral experience demonstrating innovation and excellence in their field. Candidates will be expected to possess a long-term vision of their scientific interests, to establish a vigorous and innovative research program, and to actively contribute to the Institute's mission and collegiality.

Deadline for applications is **October 31, 2014**. Applicants should submit a cover letter, a CV, a research plan and vision statement, and arrange for the submission of three letters of reference through our application page at: <http://www.stowers.org/facultysearch>. Questions should be directed to the Search Committee Chair, **Dr. Alejandro Sánchez Alvarado** (facultysearch@stowers.org).

The Stowers Institute for Medical Research is proud to be an Equal Opportunity Employer. All qualified applicants will be afforded equal opportunity regardless of race, creed, color, religion, gender, sexual orientation, pregnancy, national origin, age, disability, military status, or any other status protected by law.



Weill Cornell Medical College

Faculty and Postdoctoral Positions in Molecular Imaging Innovations Institute

The Molecular Imaging Innovations Institute (MI³) in the Department of Radiology at Weill Cornell Medical College, located in New York City, is seeking highly motivated applicants to join our newly established institute, which is located in the brand new state-of-the-art Belfer Research Building. MI³ is committed to becoming an internationally recognized institute of excellence in the discovery and development of new molecular imaging agents, and strategies for use in basic and translational research as well as in clinical care. Scientists will have access to on-site imaging systems for animal and human studies, including MRI, PET, SPECT, CT, optical and ultrasound.

Faculty positions: Experienced scientists with a PhD and/or MD degree in developing multimodality imaging strategies are encouraged to apply. Junior candidates must possess at least three years of postdoctoral experience and have publications in high impact journals. Candidates must also demonstrate the ability, or potential, to acquire external research funding. Senior positions will be considered commensurate with experience and track record of extramural funding and publications.

Postdoctoral Associate positions: The Molecular Imaging Innovations Institute seeks both chemists and biologists who are creative, motivated, and enjoy working independently as well as in a collaborative environment. Research experience in synthetic organic chemistry, medicinal chemistry, fluorescence chemistry, photodynamic therapy, peptide, liposome, nanotechnology, radiochemistry, drug delivery, immunology, stem cells or animal models are preferred, although other areas may also be considered. Applicants must have obtained a doctoral degree and possess both excellent oral and writing skills.

Qualified applicants for either position should respond by sending or emailing a letter of interest and including the following as attachments: research interests, CV, and three references to: **Dr. Ching Tung, Director of Molecular Imaging Innovations Institute, Department of Radiology, Weill Cornell Medical College, 413 East 69th Street, Mailbox 290 New York, NY 10021**; Email at MI3@med.cornell.edu. Evaluation of applications will begin immediately and continue until the positions are filled.

Weill Cornell Medical College is an employer and educator recognized for valuing AA/EOE/M/F/Protected Veterans, and Individuals with Disabilities.

Caltech

CALIFORNIA INSTITUTE OF TECHNOLOGY invites applications for tenure-track faculty positions in the Division of Chemistry and Chemical Engineering

Assistant Professor Chemistry

Areas of particular interest include inorganic chemistry, physical chemistry, chemical biology and biochemistry, although applications in any area of chemistry or biochemistry are welcomed. Exceptionally well-qualified applicants at the tenured level may also be considered. Candidates with strong commitments to research and teaching excellence are encouraged to apply. The term of the initial untenured appointment is four years, and the appointment is contingent upon completion of all requirements for a Ph.D. in chemistry, biochemistry, or in a related field.

Interested candidate should apply electronically <https://applications.caltech.edu/job/chemistry>. Candidates unable to apply electronically may submit curriculum vitae, publication list, a description of proposed research, and three letters of recommendation to: **Chair of the Chemistry Search Committee, M/C 164-30, California Institute of Technology, Pasadena, CA 91125**. Applications should be received by **October 7, 2014**.

Assistant Professor Chemical Engineering

Assistant Professor level in the area of chemical engineering. Exceptionally well-qualified applicants at the tenured level may also be considered. Candidates with strong commitments to research and teaching excellence are encouraged to apply. The term of the initial untenured appointment is four years and is contingent upon completion of all requirements for a Ph.D. in chemical engineering or in a related field.

Interested candidate should apply electronically <https://applications.caltech.edu/job/chemeng>. Candidates unable to apply electronically may submit curriculum vitae, publication list, a description of proposed research, and three letters of recommendation to: **Chair of the Chemical Engineering Search Committee, M/C 210-41, California Institute of Technology, Pasadena, CA 91125**. Applications should be received by **December 15, 2014**.

EOE of Minorities/Females/Protected Vets/Disability.



Tenure-Track Faculty Position Department of Microbiology and Physiological Systems

The Department of Microbiology and Physiological Systems at the University of Massachusetts Medical School (<http://www.umassmed.edu/>) invites applications for a tenure-track faculty position at the rank of Assistant Professor. Depending on qualifications, candidates may be proposed for a more senior appointment at the rank of Associate or Full Professor. The Department is seeking candidates who can build on its core strengths in systems biology of infectious disease, immunology, virology, and cellular physiology. We are particularly interested in candidates with cross-disciplinary approaches to problems in bacterial or viral infection including, but not limited to: integrative/systems approaches to infection; analysis of complex microbial communities and their impact on the host; molecular mechanisms of pathogenesis; cell biology of infection; and host responses and adaptation to infection. Candidates will be expected to develop and maintain an innovative, externally funded research program. We offer generous support and a highly collaborative environment with opportunities for both basic and translational research. The position will be highly competitive with regard to start-up funds and salary.

Review of applications will begin on October 1, 2014 and continue until the position is filled.

Applicants should submit a cover letter explaining their interest in the Department, a curriculum vitae that includes honors and publications, and a succinct research plan to <https://academicjobsonline.org/ajob/jobs/4663>. To expedite the review process, applicants should invite three individuals who are familiar with their work and potential for success to upload recommendation letters at the same web address.

UMass Medical School is committed to being an equal opportunity and affirmative action employer and recognizes the power of a diverse community. We encourage applications from protected veterans, individuals with disabilities and those with varied experiences, perspectives and backgrounds to consider UMass Medical School as their employer of choice.



Tenure-Track Position in Biology

The Department of Biology at University of Pennsylvania invites applicants for a tenure-track position in Neuroscience using state-of-the-art cellular or molecular genetic techniques to investigate the neural circuitry underlying complex behavior. All types of behaviors and animal models will be considered, with a special interest in behaviors that include a social, interactive component. The appointment is anticipated at the level of Assistant Professor, but exceptional senior candidates will be given serious consideration.

Penn's Department of Biology has a long-standing tradition of maintaining an integrated research and educational program across all basic biological sciences, including Molecular and Cellular Biology, Neuroscience, Genomics, Ecology and Evolution, and Plant Sciences. The Department values interdisciplinary research, collaboration, and collegiality, with a vision that emphasizes "Life in its Natural Context".

Candidates are expected to have demonstrated excellence and productivity in research, and to participate in undergraduate and graduate teaching. Interested candidates should submit materials online at <http://facultysearches.provost.upenn.edu/postings/321> and include a curriculum vitae, concise statements of research and teaching interests, a short annotated description of up to five publications with the most impact and/or creativity, and the names with contact information of at least three referees whom we will contact for an appraisal. Review of applicants will begin **November 1, 2014** and continue until the position is filled.

The Department of Biology is strongly committed to Penn's Action Plan for Faculty Diversity and Excellence and to creating a more diverse faculty (for more information see: <http://www.upenn.edu/almanac/volumes/v58/n02/diversityplan.html>). The University of Pennsylvania is an EOE. Minorities/Women/Individuals with disabilities/Protected Veterans are encouraged to apply.



Assistant Professor

The Department of Mechanical and Aerospace Engineering (MAE) at Princeton University is conducting a broad search for two (2) tenure-track assistant professors. We welcome applications from all areas in mechanical and aerospace engineering, including but not limited to the fields of robotics, lightweight structures, nonlinear mechanics, engineering systems, propulsion, and energy systems and efficiency. Applicants must hold a Ph.D. in Engineering, Materials Science, Physics, or a related subject, and have a demonstrated record of excellence in research with the potential to establish an independent research program. We seek faculty members who will create a climate that embraces excellence and diversity, with a strong commitment to teaching and mentoring.

Princeton's MAE department has a long history of leadership in its core areas of Applied Physics, Dynamics and Controls, Fluid Mechanics, Materials Science, and Propulsion and Energy Sciences, with additional strength in cross-disciplinary efforts impacting areas such as biology, the environment, security, and space. We seek creative and enthusiastic candidates with the background and skills to build upon and complement our existing departmental strengths and those who can lead the department into new and exciting research areas in the future.

To ensure full consideration, applications should be received by **December 1, 2014**. Applicants should submit a curriculum vitae, including a list of publications and presentations, a 3-5 page summary of research accomplishments and future plans, a 1-2 page teaching statement, and contact information for at least three references online at <http://jobs.princeton.edu>, requisition number 1400675. Personal statements that summarize leadership experience and contributions to diversity are encouraged.

Princeton University is an Equal Opportunity Employer and all qualified applicants will receive consideration for employment without regard to race, color, religion, sex, national origin, disability status, protected veteran status, or any other characteristic protected by law. We welcome applications from members of all underrepresented groups. This position is subject to the University's background check policy.



FACULTY POSITION IN BIOCHEMISTRY, MOLECULAR BIOLOGY, AND GENETICS

The Department of Biochemistry and Molecular Biology at the Penn State University College of Medicine is expanding under the new leadership of Dr. James R. Broach. The Department invites applications from outstanding scientists with Ph.D., M.D., or equivalent degrees for a full-time tenure-track position. We seek candidates at the Assistant Professor level who have an active highly competitive independent research program or who show a strong potential to develop such a program. We are looking for candidates in the areas of molecular biology, genetics, epigenetics, and/or genomics. Candidates will have the opportunity to participate in Penn State's medical genomics program through the new Institute for Personalized Medicine.

For additional information, please visit the following website:
<http://www2.med.psu.edu/biochemistry/>

Applicants should submit a curriculum vitae and a brief statement of research plans to www.psu.edu, position # and arrange for three letters of reference to be sent to Faculty Search Committee, biochem_apply@hmc.psu.edu or to Department of Biochemistry and Molecular Biology H171, Penn State University College of Medicine, Hershey PA 17033. Application should be received prior to **November 15, 2014**.

Penn State is committed to Affirmative Action, Equal Opportunity and the diversity of its workforce.



Faculty Positions in the Center for Autophagy Research

The Center for Autophagy Research at the University of Texas Southwestern (UTSW) Medical Center is a newly created Center designed to foster a collaborative interdisciplinary environment for promoting cutting-edge research in the field of autophagy. The Center is seeking new faculty members at the Assistant Professor (tenure track) level. Faculty will be expected to develop state-of-the-art, innovative independent research programs related to the biochemistry, structural biology, cell biology, genetics, pharmacology, developmental biology or physiological/pathophysiological roles of autophagy. Excellent opportunities exist for collaborations with faculty members in basic science and clinical Departments at UTSW. UTSW is an outstanding scientific environment with established strengths in structural biology, biochemistry, cell biology, microbiology, molecular biology, genetics, and numerous other areas. The positions offer attractive start-up packages and laboratory space. Candidates should have an M.D. and/or a Ph.D. degree with at least 3-4 years of postdoctoral experience and an outstanding publication record.

To apply, submit a C.V., three letters of reference, and a description of research interests to:

Dr. Beth Levine, Director
Center for Autophagy Research
UT Southwestern Medical Center
5323 Harry Hines Blvd,
Dallas, TX 75390-9113
E-mail: Haley.Harrington@UTSouthwestern.edu

UT Southwestern is an Equal Opportunity/Affirmative Action Employer.



**THE UNIVERSITY OF TEXAS
AT DALLAS**
The School of
Natural Sciences and Mathematics

BIOLOGICAL SCIENCES OPEN RANK FACULTY POSITIONS

The Department of Biological Sciences (formerly Molecular and Cell Biology) in the School of Natural Sciences and Mathematics at the University of Texas at Dallas invites applications for **Faculty Positions** at any rank. Applications are particularly encouraged from evolutionary biologists conducting research that complements and builds on existing Departmental strengths in biochemistry, cancer biology, cell biology, computational and systems biology, microbiology, and neurobiology. Examples of specific research areas of interest include (but are not limited to): evolutionary developmental biology, molecular evolution, human population genetics, evolution of infectious diseases, evolutionary genomics, evolutionary cell biology, evolutionary computational biology, and evolutionary medicine (especially relating to cancer). We will also consider applications from outstanding candidates in other areas of the biological sciences.

Applicants should show evidence of a vigorous and independent research program, and those at the Associate or Full Professor levels should also have a record of obtaining significant external funding. Applicants should have enthusiasm for teaching at both graduate and undergraduate levels.

Review of applications will begin immediately and will continue until the positions are filled. Indication of gender and ethnicity for affirmative action statistical purposes is requested as part of the application.

Application materials (curriculum vitae, short descriptions of research plans and teaching interests, and three letters of recommendation) should be submitted via the **ONLINE APPLICATION FORM** available at <http://go.utdallas.edu/pnp140912>

The University of Texas at Dallas is an Equal Opportunity/Affirmative Action Employer. All qualified applicants will receive consideration for employment without regard to race, color, religion, sex, national origin, disability, pregnancy, age, veteran status, genetic information or sexual orientation.



**University of
Zurich** UZH

Faculty of Medicine

The Faculty of Medicine at the University of Zurich invites applications for an

Assistant Professorship non-tenure track (3 + 3 years) with a focus on Tumor Genomics within the University Research Priority Program «Translational Cancer Research»

We seek a scientist or physician scientist in the field of tumor genomics and bioinformatics with a documented excellent international track-record and the capacity to build a dynamic and integrative research program. The successful candidate will have a strong interest and background in interdisciplinary cancer research and in linking basic and clinical research, and ideally combines computational and experimental approaches. Experience and demonstrated excellence in one or more of the following research areas is desirable: assembly and mining of large datasets, whole genome sequence analyses, functional cancer genomics, network analyses, translational cancer research. Applicants for this function must hold a PhD, MD or equivalent degree in the life sciences and should have three or more years of relevant post-graduate research experience; clinical duties are not associated with this position.

The Assistant Professor will be affiliated with the Medical Faculty and with the Faculty of Science of the University of Zurich, and will be part of the Cancer Network Zurich, the Cancer Biology PhD program and the above-mentioned URPP. We offer a competitive start-up package, salary and laboratory space at the research campus of the University of Zurich.

Our URPP aims to connect basic, translational and clinical cancer research and consists of a consortium of 11 principal investigators, who are leaders in the field of cancer research (www.cancer.uzh.ch). The University of Zurich is an equal opportunities employer.

Please submit your application (in duplicate plus one CD) by October 31st 2014 to the Dean's Office, Faculty of Medicine, Pestalozzistrasse 3-5, CH-8091 Zurich. For further information, please contact the President of the Search Committee, Prof. Dr. Anne Müller, e-mail: mueller@imcr.uzh.ch.

The application should be prepared according to the «Instructions for the submission of applications at the Faculty of Medicine, University of Zurich»: see <http://www.med.uzh.ch/FormulareundRichtlinien/Bewerbung.html>

PURDUE UNIVERSITY

Faculty Position in Developmental Biology Department of Biological Sciences

We invite applicants for a tenure-track faculty position in Developmental Biology. We welcome colleagues whose research activities will complement our existing focus areas in neurosensory systems, neurodegeneration, cancer and other diseases, epigenetics or pathogenesis. Potential model systems of interest are mouse, zebrafish, fruit fly or stem cells to study development and disease. The successful applicant is expected to address fundamental questions in developmental biology using varied experimental approaches such as advanced imaging, biochemistry, genomics, genetics and/or gene transfer for translational studies. The position requires teaching graduate and undergraduate courses, mentoring research students and contributing to the mission of the department.

Applicants must have a Ph.D. in Biological Sciences or related discipline and at least 2 years of postdoctoral experience. We expect to fill academic year appointments at the Assistant Professor level, but appointments at the Associate level will be considered.

The Department has over 50 faculty conducting research in a wide range of fields (www.bio.purdue.edu/). We are committed to the success of all new faculty: we assign faculty mentors and offer competitive startup packages. Opportunities abound to use advanced research facilities across campus, such as the Bindley Bioscience Center (www.purdue.edu/discoverypark/bioscience/).

Applications must be submitted electronically to <http://hiring.science.purdue.edu> as a PDF with a detailed curriculum vitae, contact information for three referees, a 2-3 page summary of research interests and a teaching statement. A clear description of your major research accomplishments and future plans is more valuable for our evaluation than the impact factors of the journals in which you have published. Direct inquiries to search@bio.purdue.edu. Review of applications will begin **October 1, 2014** and continue until the position is filled. A background check will be required for employment in this position. Purdue University, West Lafayette, IN is a dual career friendly employer.

Purdue University is an EEO/AA Employer. All individuals, including minorities, women, individuals with disabilities, and protected veterans are encouraged to apply.

WAYNE STATE UNIVERSITY

Tenured/Tenure Track Faculty Position in Biological Sciences

The Department of Biological Sciences at Wayne State University (<http://www.clas.wayne.edu/biology/>) is searching for one tenure-track faculty member specializing in **systems biology** or **microbiology**. Rank is open depending upon qualifications. The systems biologist may work at the molecular, cellular, organismal or community level in areas complementing the department's existing strengths in development, neurobiology, transcription, evolution or ecology. Areas of interest in microbiology include, but are not limited to, bacteriology, virology, immunology, host-pathogen interactions, environmental microbiology or infectious disease processes.

Wayne State University is a large, comprehensive, nationally ranked research institution that offers state-of-the-art research facilities and highly competitive start-up packages. The metropolitan Detroit area offers a rich cultural and educational environment, an excellent standard of living, and easy proximity to Michigan's lakes, forests and recreational sites. Applicants must have a Ph.D. degree, postdoctoral experience and an outstanding record of research achievement. Successful applicants are expected to establish and maintain vigorous, externally funded research programs and to participate in graduate and undergraduate education. Please apply on-line at jobs.wayne.edu. In addition to the online application that includes cover letter and curriculum vitae, applicants must submit a 2-page statement of their research plans and have three letters of reference sent directly to the Faculty Search Committee: ad5348@wayne.edu. Please apply by **November 15, 2014** for full consideration. Applications will be considered only when all materials have been received.

Wayne State University is an Affirmative Action/Equal Opportunity Employer. Women and members of minority groups are especially encouraged to apply.

UNIVERSITY OF MICHIGAN

Ecology or Evolutionary Biology of Fishes or Birds

The Department of Ecology and Evolutionary Biology (www.lsa.umich.edu/eeb) and the Program in the Environment (www.lsa.umich.edu/pite) at the University of Michigan seek applicants for an assistant professor (tenure-track) position in the ecology or evolutionary biology of fishes or birds. While we expect to make a junior hire, outstanding senior applicants will also be considered. This is a university-year appointment with an expected start date of September 1, 2015. We seek outstanding individuals who use comparative fish or bird systems to study any area of ecology or evolutionary biology, and who would offer exceptional courses in the ecology or evolution of either taxon. Also strongly encouraged are research programs that could take advantage of the world-class biodiversity collections of the Museum of Zoology and/ or utilize the EEB Department's biological field stations. Museum curatorial activities may replace some teaching duties for appropriate candidates.

To apply, use this link - www.resources-eeb.lsa.umich.edu/search14 - and arrange to have three letters of recommendation submitted through the same website. Review of applications will begin on **November 3rd 2014** and will continue until the position is filled.

Women and minorities are strongly encouraged to apply. The University of Michigan is supportive of the needs of dual career couples and is an Equal Opportunity/Affirmative Action Employer.

2014 Annual Top Employers in Biotech & Pharma

For recruitment in science, there's only one

Science



Special Career Feature: October 17

Ads accepted until October 10 if space is still available.

Who is No. 1 this year?

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Science Careers

From the journal Science

AAAS

ScienceCareers.org

Assistant/Associate/Full Professor

Small fruit or vegetable genomics/breeder

100% Research 12 month, tenure track

Location: Plants for Human Health Institute
North Carolina State University
North Carolina Research Campus
Kannapolis, North Carolina 28081

Position Available: March 1, 2015

Qualifications:

Ph.D. degree in an appropriate field of study and documented background in plant breeding, molecular genetics and/or applied genomics. This position will contribute research that will target the health-promoting, phytoactive compounds inherent in small fruits and berries, and/or vegetable produce, and investigate strategies for selecting for, concentrating and preserving these phytochemicals. It is essential that the incumbent conduct team-oriented, transdisciplinary research, exhibit exceptional leadership abilities, and demonstrate effective written and verbal communication skills. Postdoctoral experience is preferred.

Responsibilities:

The successful candidate's research will focus on genetics, genomics, germplasm improvement and breeding of small fruits (strawberries, blackberries, raspberries, blueberries, etc.) and/or vegetable crops. Expertise in ploidy manipulation and interspecific hybridization is desirable. The faculty member is expected to lead a genomics program in small fruits and/or vegetables. The incumbent will lead an active varietal breeding program that includes a strong field component. The successful candidate will be expected to interface and collaborate with PPHI team scientists who specialize in phytochemical characterization, pharmacogenomics, genomics, metabolomics, systems biology, and post-harvest physiology. Advising and mentoring of graduate students is expected. Candidate will be placed in an appropriate home department in the College of Agriculture and Life Sciences (CALS) at NCSU depending on interests and expertise. The successful candidate will be expected to collaborate with other academic partners (8 universities located on the NCRC) as well as extension professionals.

Application:

Applicants should apply online at <https://jobs.ncsu.edu/postings/41985> by Nov 15, 2014.

NW252894R



UNIVERSITY of WASHINGTON

Faculty Position Eukaryotic Cell Biology Department of Biology

As part of a long-term strategy to enhance strengths in Cell Biology, the Department of Biology is searching to hire a full-time (9-month) Assistant Professor (Job class 0116) for a tenure-track faculty position in eukaryotic cell biology. We seek candidates who integrate perspectives from multiple disciplines, use quantitative approaches, and appreciate the breadth of research encompassed within the Department. We are especially interested in candidates using experimentally tractable plant, animal or protist systems to investigate fields including but not limited to cell homeostasis, signaling, polarity, proliferation, motility, membrane trafficking, interactions between cells and their environments, developmental cell biology, and evolutionary cell biology.

We are looking for individuals with a record of outstanding achievement or strong indications of outstanding future potential. Priority will be given to applications received by **3 November 2014** at: <http://www.biology.washington.edu/faculty/search/>. Applicants must have earned a doctorate by the date of appointment. All University of Washington faculty engage in teaching, research, and service.

The University of Washington is an Affirmative Action, Equal Opportunity Employer. All qualified applicants will receive consideration for employment without regard to, among other things, race, religion, color, national origin, sex, age, status as protected veterans, or status as qualified individuals with disabilities. The University is building a culturally diverse faculty and staff and strongly encourages applications from women, minorities, individuals with disabilities and covered veterans. The University is the 2006 recipient of the Alfred P. Sloan award for Faculty Career Flexibility, and is committed to supporting the work-life balance of its faculty. Our NSF-supported ADVANCE program <http://advance.washington.edu/> is dedicated to increasing the participation of women in STEM disciplines.



WISCONSIN
UNIVERSITY OF WISCONSIN-MADISON

Faculty Positions in Biodesign and Biocatalysis

Breakthroughs in systems biology, computational biology, and synthetic biology coupled with advances in genomics and rational design have opened dramatic opportunities to create microbes and plants for sustainable bioenergy production and other applications. Similarly, new approaches to the design of biological, bio-inspired, and chemical catalytic systems promise low-cost, renewable, and sustainable catalysts with applications to biofuels, chemicals, and societal needs. To promote scientific advances in these areas and as part of a commitment to improve and diversify how energy is provided for human needs, UW-Madison in association with the Wisconsin Energy Institute invites applications for new faculty of any rank to develop significant research programs in biodesign or biocatalysis. Applicants with experience or interest in cross-disciplinary research and collaboration as well as teaching and mentoring will be especially competitive.

Anticipated tenure and research program homes include:

- Bacteriology
- Biochemistry
- Biological and Chemical Engineering
- Chemistry
- other relevant UW departments

Applicants with a PhD and strong record of achievement should apply by sending a single pdf containing a cover letter referencing pvl 80828, a curriculum vitae with summary of research accomplishments, and a statement of future research program and teaching interests to facultysearch@energy.wisc.edu. Applicants should arrange for letters of recommendation from three references to be sent to the same address. For full consideration, applications should be submitted by **December 1, 2014**.

The University of Wisconsin-Madison is an Equal Opportunity/Affirmative Action Employer. We promote excellence through diversity and encourage all qualified individuals to apply.

POSITIONS OPEN



**ELI-HU Research and Development
Non-Profit Limited Liability Company
is looking for
DIVISION HEAD FOR THE SCIENTIFIC
APPLICATION DIVISION OF THE ELI-
ALPS RESEARCH INFRASTRUCTURE**

The ELI Attosecond Light Pulse Source (ELI-ALPS)

research centre to be built in Szeged (Hungary) will be devoted to the study of ultrafast dynamics on the femto/attosecond timescale in atoms, molecules, plasmas and biological samples.

The Division Head is expected to be the driving force in the definition/selection of the research topics/activities and the formation/coordination of the research groups of the division that will pursue research in a broad spectrum of high harmonics, fast particle and laser applications at ELI-ALPS.

You are welcome to **submit your application** along with your detailed CV in English to allas@eli-alps.hu



SZÉCHENYI 2020



INVESTING IN YOUR FUTURE



Hunting for Talents NUAA, Jiangsu, China

Nanjing University of Aeronautics and Astronautics (NUAA) is a research-oriented national key university of "211 Project". It also enjoys a well-balanced development of multiple disciplines in engineering, technology, natural sciences, economy, management and social sciences with the characteristics of aeronautics, astronautics and civil aviation. NUAA is qualified to be "Dominant Discipline Innovation Platform of 985 Project" and to independently recruit and receive international students who are granted the Chinese Government Scholarship. Now NUAA consists of 16 colleges with more than 3,000 staff members and approximately 26,000 degree students.

Academia and education at NUAA represent strong capacity among all the universities in China. It has acquired national status through the quality of its excellence research work, especially in the areas of Aerospace Engineering, Mechanics, Electromechanics, Economy and Management, etc.

NUAA gives a warm welcome to excellent experts, scholars and young students from both home and abroad, who are willing to serve the country, dedicate themselves to the development of aerospace science and make contributions to the industrialization, information technology of China. NUAA will provide teachers and researchers with a good academic environment, satisfactory working and living conditions and a stage on which they can put their talents to good use.

Contacts

Ms. Zhao Haiyan, Mr. Cao Yunxing

Personnel Division, NUAA

Address: 29# Yudao St. Nanjing, Jiangsu Province, Postcode: 210016

Tel: +86-25-84892461

Fax: +86-25-84895923

Email: zhaohaiyan@nuaa.edu.cn

Web: <http://www.nuaa.edu.cn/nuaanew> <http://rsc.nuaa.edu.cn>



武汉大学

Faculty Positions Available at The IAS and The MRI, Wuhan University, Wuhan, China

Two newly founded institutes at Wuhan University in China, the Institute for Advanced Studies (IAS) and the Medical Research Institute (MRI), cordially invite applications for ~50 each, open-rank faculty positions in Biology, Chemistry, Physics, Material Sciences, and Medical Sciences.

All applicants must have a Ph. D or MD and a successful postdoctoral experience. Successful candidates will be expected to establish an active research program in relevant disciplines. We offer internationally competitive recruitment packages.

The applicants should submit, electronically, a full CV, a research statement and contact information of three referees in a single PDF file to wgdgy@whu.edu.cn (for IAS positions) or shuoffice@whu.edu.cn (for MRI positions).

Applications that apply for both institutes at the same time will not be accepted and further processed.

<http://hr.whu.edu.cn/>



西南交通大学
Southwest Jiaotong University

Southwest Jiaotong University, P.R.China
Anticipates Your Working Application

Southwest Jiaotong University (SWJTU), founded in 1896, situates itself in Chengdu, the provincial capital of Sichuan. It is a national key multidisciplinary "211" and "985 Feature" Projects university directly under the jurisdiction of the Ministry of Education, featuring engineering and a comprehensive range of study programs and research disciplines spreading across more than 20 faculties and institutes/centers. Boasting a complete Bachelor-Master-Doctor education system with more than 2,500 members of academic staff, our school also owns 2 first-level national key disciplines, 2 supplementary first-level national key disciplines (in their establishment), 15 first-level doctoral programs, 43 first-level master programs, 75 key undergraduate programs, 10 post-doctoral stations and more than 40 key laboratories at national and provincial levels.

Our university is currently implementing the strategy of "developing and strengthening the university by introducing and cultivating talents". Therefore, we sincerely look forward to your working application.

More information available at <http://www.swjtu.edu.cn/>

I. Positions and Requirements

A. High-level Leading Talents

It is required that candidates be listed in national top talents programs such as *Program of Global Experts*, *Top Talents of National Special Support Program*, *"Chang Jiang Scholars"*, *China National Funds for Distinguished Young Scientists* and *National Award for Distinguished Teacher*.

Candidates are supposed to be no more than 50 years old. The limitation could be extended in the most-needed areas of disciplinary development.

Candidates who work in high-level universities/institutes and reach the above requirements are supposed to be no more than 45 years old.

B. Young Leading Scholars

Candidates are supposed to be listed in or qualified to apply for the following programs:

• National Thousand Young Talents Program

• The Top Young Talents of National Special Support Program (Program for Supporting Top Young Talents)

• Science Foundation for the Excellent Youth Scholars

Candidates should have good team spirit and leadership, outstanding academic achievements, broad academic vision and international cooperation experience and have the potential of being a leading academic researcher.

C. Excellent Young Academic Backbones

Candidates under 40 years old are expected to graduate from high-level universities/institutes either in China or other countries. Those who are professors, associate professors and other equal talents from high-level universities/institutes overseas could be employed as professors and associate professors as well.

D. Excellent Doctors and Post Doctoral Fellows

Candidates under 35 years old are supposed to be excellent academic researchers from high-level universities either in China or other countries.

II. Treatments

The candidates will be provided with competitive salaries and welfares that include settling-in allowance, subsidy of rental residence, start-up funds of scientific research, assistance in establishing scientific platform and research group as well as international-level training and promotion. As for outstanding returnees, we can offer further or specific treatments that can be discussed personally.

III. Contact us:

Contacts: Ye ZENG & Yinchuan LI Telephone number: 86-28-66366202 Email: talent@swjtu.edu.cn

Address: Human Resources Department of SWJTU, the western park of high-tech zone, Chengdu, Sichuan, P.R.China, 611756

<http://www.swjtu.edu.cn/>



Hiring Professors at All Ranks at South University of Science and Technology (SUSTC) Shenzhen, China

The South University of Science and Technology (SUSTC) invites applications and nominations for all ranks of tenured and tenure-track faculty members in the Division of Science, Division of Engineering and Division of Management & Finance.

SUSTC, officially established in April 2012, is a public institution funded by the municipal of Shenzhen, a special economic zone city in southern China. The University is accredited by the Ministry of Education, China and is a pioneer in higher education reform in China. Set on five hundred acres of wooded landscape in the picturesque Nanshan (South Mountain) area, the new campus offers an idyllic environment suitable for learning and scholarship. SUSTC engages in basic and problem-solving research of lasting impact to benefit society and mankind.

The Division of Science, Division of Engineering, and the Division of Management & Finance wish to hire faculty members at all ranks. Key areas include but not limited to: *Neural and Cognitive Sciences, Biology and Gene Engineering, Modern Physics, Control and Modification of Materials, Nanoscience and Nanotechnology, Mathematics and Applied Mathematics, Molecular Chemistry and Catalysis, Large-Scale Computational Research, Robotics and Artificial Intelligence, Information Systems and Electronic Engineering, Modern Cities and Future Developments, Energy Sciences and Technology, Environmental Sciences, Financial Mathematics and Management Sciences*. The Divisions especially encourage research that requires a multi-disciplinary approach. Experienced researchers whose interests do not fall within the above areas are invited to suggest new areas of research. **Cluster hiring is possible, with senior members accompanied by junior members in a group.**

The teaching language at SUSTC is English or Putonghua. The choice is made by the instructor. As we expect an international faculty, the majority of teaching materials and reference books will be in English and many classes will be conducted in English. With a very high faculty-to-student ratio, SUSTC is committed to delivering a student-centered education and encourages students to develop their innovative spirits. Students at junior and senior years are expected to participate in research in the Research Centers.

The University offers competitive salaries, fringe benefits including medical insurance, retirement and housing subsidy. Leading Professors, Chair Professors and Professors will be appointed with tenure. Associate Professors and Assistant Professors will be offered tenure-track contracts.

Please visit our website to apply: <http://talent.sustc.edu.cn/en/>. All applications should include a CV and a detailed list of publications with Research ID. **Those interested in cluster hiring should send CVs and publication lists with Research ID as a group.** Evaluations will commence immediately and appointments will be made on a continuous basis. Additional information on SUSTC is available on the University homepage <http://www.sustc.edu.cn>.

Qualified applicants are also encouraged to apply for the Recruitment Program of Global Expert ("Thousand Talents Program") through SUSTC. Successful applicants will get extra research fund and living allowance from the government. Additional information is available through email inquiry or <http://talent.sustc.edu.cn/>.

If you have any questions, please feel free to contact us at hiring@sustc.edu.cn.

FACULTY POSITIONS



ASSOCIATE OR FULL PROFESSOR NCI designated Sidney Kimmel Cancer Center Prostate Cancer Program Thomas Jefferson University

Applications are invited for a tenure-track appointment as either an Associate or full Professor in the Prostate Program of the NCI-designated Sidney Kimmel Cancer Center at the Thomas Jefferson University. Cancer Center members within the Prostate Program have diverse research interests that include molecular genetics and cell signaling, chromatin and gene regulation, hormone action, metastasis, DNA damage and repair, radiation oncology, medical oncology, and surgical oncology.

The Prostate Program seeks accomplished scientists or physician scientists (Ph.D., M.D., or M.D.-Ph.D.) with well-established, innovative research programs associated with current clinical challenges in prostate cancer management. For exceptional candidates, leadership opportunities will be considered. *Jefferson values diversity and encourages applications from women, members of minority groups, LGBTQ individuals, disabled individuals, and veterans.* In a single PDF document, applicants should submit curriculum vitae, a brief statement of past research accomplishments and future research interests, description of leadership experience, and list three references. Applications should be sent electronically to the Kimmel Cancer Center Prostate Cancer Program Search Committee, c/o e-mail: elizabeth.schade@jefferson.edu.

FACULTY POSITION in Complex Systems

College of Engineering & Mathematical Sciences

The University of Vermont (UVM) the College of Engineering and Mathematical Sciences invites applications for a tenure-track faculty position in Complex Systems, with a focus on research problems in the behavioral and social sciences. The position is in support of our NSF-funded IGERT program on Smart Grid & Human Behavior. Requirements include an earned doctorate in computer science, mathematics, or a related discipline, a proven record of scholarly activities, and the qualifications to teach both undergraduate and graduate courses in their home department. The full job description and online application can be found at [website: http://www.cems.uvm.edu/facsearch/csyzs.php](http://www.cems.uvm.edu/facsearch/csyzs.php). The first review of applications will occur on December 15, 2014. *UVM is an Equal Opportunity/Equal Access/Affirmative Action Employer and conducts background checks on all final candidates.*

 **More
scientists
agree—we
are the most
useful website.**

Science Careers

From the journal *Science* 

ScienceCareers.org

POSTDOCTORAL OPPORTUNITIES



UNIVERSITY OF
SOUTH CAROLINA
School of Medicine

POSTDOCTORAL FELLOWSHIPS

Postdoctoral fellowships are available to pursue research supported by NIH grants including the Center of Excellence for Complementary and Alternative Medicine on Autoimmune and Inflammatory Diseases ([website: http://camcenter.med.sc.edu/](http://camcenter.med.sc.edu/)). Studies are aimed at examining the effects of plant products such as resveratrol, indoles, and cannabinoids on inflammation, autoimmunity, and cancer. Ph.D. in a relevant area is required. Experience required in extraction of plant products, isolation, and characterization of bioactive compounds or 'omics/microbiome technology. Send curriculum vitae to: **Dr. Mitzi Nagarkatti, Carolina Distinguished Professor and Chair, Department of Pathology, Microbiology and Immunology, University of South Carolina School of Medicine, Columbia, SC29229** or e-mail: postdocmedchem@uscm.edu. *USC Columbia is an Equal Opportunity/Affirmative Action Employer and encourages applications from women and minorities.*

FACULTY POSITIONS

NEUROSCIENCE FACULTY POSITION Fordham University

We are accepting applications for a tenure-track position at the ASSISTANT/ASSOCIATE PROFESSOR level in the Biology Department. She or he will have an outstanding research program and publication record. Commitment to excellence in teaching and mentoring is required. The recruit will be expected to cover the area of cellular-molecular neuroscience in research and teaching, and contribute to the new interdisciplinary Integrative Neuroscience major and the Department's expanding graduate M.S. and Ph.D. programs. She or he is expected to establish (for Assistant level) or continue (for Associate level) a research program supported by external grants that offers opportunities for mentored research by graduate and undergraduate students. The Department provides excellent research facilities, startup funds, and competitive salaries and benefits. Applicants should electronically send one PDF application file containing a cover letter, curriculum vitae, contact information for three references, research and teaching statements, and three reprints to e-mail: jdlewis@fordham.edu. Address the cover letter to: **Dr. J.D. Lewis, Chair, Department of Biological Sciences, Fordham University, Bronx, NY 10458**. Review of applications will begin October 15, 2014. *Fordham University is an independent, Catholic university in the Jesuit tradition that welcomes applications from men and women of all backgrounds. Fordham University is committed to excellence through diversity and welcomes candidates of all backgrounds; it is an Equal Opportunity Employer.*

COMPUTATIONAL BIOLOGY FACULTY POSITIONS School of Computer Science Carnegie Mellon University

We seek outstanding researchers who are developing computational methods in all areas of biology for tenure-track and research-track positions at all levels in the Lane Center for Computational Biology ([website: http://lane.compbio.cmu.edu](http://lane.compbio.cmu.edu)), a department within our School of Computer Science. Appointments will be made either entirely in the Lane Center or jointly with other departments in the university, as appropriate to the background and interests of the candidate. We especially seek candidates working in biological and medical imaging, genomics, and those using advanced machine learning methods to analyze measurements of complex phenomena. For further information, see [website: http://lane.compbio.cmu.edu/positions-available](http://lane.compbio.cmu.edu/positions-available). *Carnegie Mellon is an Equal Employment Opportunity/Affirmative Action Employer—Minorities/Females/Persons with Disability/Veteran.*



Nontraditional Careers: Opportunities Away From the Bench Webinar

Want to learn more about exciting and rewarding careers outside of academic/industrial research? View a roundtable discussion that looks at the various career options open to scientists and strategies you can use to pursue a nonresearch career.

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Science Careers

From the journal *Science* 

Careers in Neuroscience

October 31, 2014

Reserve ads by October 14
to guarantee space.

Ads accepted until October 27
if space is still available.



There's only one **Science**

This feature examines the areas and regions where the career possibilities are expanding and offers advice for scientists building their career in neuroscience.

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Reach: Your job ad is seen by 570,400 readers around the globe

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Society for Neuroscience
15–19 November
Washington, DC

To book your ad:
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Science Careers

From the journal *Science*



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Institut Pasteur

Call for projects 2015

Creation of new research groups at Institut Pasteur

The Institut Pasteur has launched an international call for candidates wishing to establish new independent research groups in the cutting edge interdisciplinary environment of its campus in Paris, France.

The Institut Pasteur is a non-profit private foundation dedicated to fundamental, interdisciplinary research and to the translation of the knowledge to medicine and public health. Topics of interest include microbiology (bacteria, viruses, parasites and fungi) and infectious diseases, immunology, developmental biology and stem cells, neuroscience, genomics, genetics and cancer.

The Institut Pasteur is now initiating a new recruitment campaign, with attractive packages for junior and mid-career scientists. Senior investigator candidates are also welcome to apply.

Successful **junior candidates**¹ will be appointed with a permanent position, and as head of a group of 6 people. These groups will be created for a period of 5 years and may thereafter compete for a full research group.

Successful **mid-career and senior candidates** will be appointed with a permanent position, and as head of a research group of 8 to 15 people. The groups will be created for 10 years (mid-term evaluation at 5 years) with the possibility of renewal.

Highly attractive packages to match the experience of the candidate will be provided, including institutional salaries (Principal investigator, permanent scientists, technician, secretary, post-doctoral fellows), a substantial contribution to running costs and equipment, access to on campus state-of-the-art technology core facilities, as well as support for relocation expenses and administrative issues.

We request a Letter of Intent (LOI) in advance of submitting a full grant application. The template can be downloaded from the Institut Pasteur website: http://www.pasteur.fr/recherche/cfp2015_loi.doc.

A pdf copy of the LOI should be electronically submitted to CFP2015@pasteur.fr no later than **Friday, November 28, 2014 by 5:00 pm** (Central European Time).

Shortlisted applicants will be notified by e-mail by mid-January 2015.

A complete application will be requested and due for submission by the end of February 2015. Applicants will be invited for interview to take place in mid-April 2015. The final ranking will be established by the Pasteur Scientific Council during its June 2015 session.

Contacts:

Practical aspects: CFP2015@pasteur.fr

Scientific aspects: alain.israel@pasteur.fr

¹Institut Pasteur is an equal opportunity employer. Junior group leaders should be less than 8 years after PhD at the time of their LOI submission. Women are eligible up to 11 years after their PhD if they have one child, and up to 14 years after their PhD if they have two or more children.

MONTEREY BAY AQUARIUM RESEARCH INSTITUTE

2015 POSTDOCTORAL FELLOWSHIP PROGRAM

Applications for the postdoctoral fellowship program at the Monterey Bay Aquarium Research Institute (MBARI) are currently being accepted. MBARI is dedicated to the development of state-of-the-art instrumentation, systems, and methods for scientific research in the oceans. Ongoing programs in marine robotics, ocean physics, chemistry, geology, and biology as well as information management and ocean instrumentation research and development exist at MBARI. Located in Moss Landing, California at the head of Monterey Canyon, MBARI enjoys convenient access to diverse oceanographic environments. The institute operates research vessels equipped with remotely operated vehicles, autonomous underwater vehicles, and diverse oceanographic equipment. In addition, MBARI operates the MARS seafloor cabled observatory. MBARI is a non-profit oceanographic research institute supported by the David and Lucile Packard Foundation.

Offers will be made to candidates from the fields of biological, chemical, and physical oceanography; marine geology; and ocean engineering. Candidates must be awarded the Ph.D. degree prior to commencing the two-year appointment and start during the 2015 calendar year. Applicants are encouraged to communicate with potential research sponsors at MBARI for guidance on project feasibility, relevance to ongoing research projects, and resource availability (http://www.mbari.org/about/postdoc_mentors.htm).

Application deadline: Wednesday, December 10, 2014

Selected candidates will be contacted in early March 2015.

Application requirements:

1. Curriculum vitae
2. At least three professional letters of recommendation
3. Succinct statement of the applicant's doctoral research
4. Potential research goals at MBARI
5. Supplemental information online form (http://www.mbari.org/oed/jobs/forms/postdoc_form_2015.html)

Address your application materials to:

MBARI, Human Resources **Job code: Postdocs-2015**
7700 Sandholdt Road, Moss Landing, CA 95039-9644

Submit by e-mail to: jobs_postdocs@mbari.org (preferred),
by mail, or fax to (831) 775-1620.

MBARI is an equal opportunity and affirmative action employer. MBARI considers all applicants for employment without regard to race, color, religion, sex, national origin, age, disability, or covered veteran status in accordance with applicable federal, state, and local laws. Competitive compensation and benefits package.



EOE • MBARI Welcomes Diversity

By David A. Anderson

A detour for love

My advisers would have told you that I had a bright future. As an undergraduate at New College of Florida, I pursued research in coral reef biology. I studied abroad in Ireland for practical training in biotechnology. I learned Spanish to aid in collecting samples for my thesis on coral immunogenetics in Honduras. As a graduate student in the Department of Marine Sciences at the University of Puerto Rico (UPR), Mayagüez, I traveled across the Caribbean monitoring coral disease outbreaks and their impacts on important reefs. I had become globalized: My personal and scientific relationships spanned continents and hemispheres. My science career seemed assured. And then I fell in love.

For lesbian, gay, bisexual, and transgender couples, love can be complicated. When the love spans nationalities, the challenges are especially formidable. I met my Chilean partner in 2009, during a Thanksgiving barbecue on the beach. He was a chemical engineering graduate student at UPR.

For the first year of our relationship, we were like any other graduate student couple. However, in 2010, we learned that UPR's accreditation was at risk after strikes that paralyzed the state university system. Many graduate students and faculty, including my advisers, were applying for positions at other universities. My partner and I followed suit, applying to programs in the United States. We were lucky to be accepted to the same university.

I am a U.S. citizen and would have married my partner to confer immigration rights. However, under the Defense of Marriage Act (DOMA), such rights were not conferred to same-sex couples, and evidence of any serious relationship with a U.S. resident would have been grounds for denial of a student visa. So my partner went back to Chile, waited for paperwork from the new university, and scheduled an appointment at the U.S. embassy. I stayed in the United States, working part time as a tutor. We told ourselves we would be separated for only a few months.

My partner's visa application was rejected. My parents had sponsored him as a "friend of the family," but the authorities didn't believe my family would be willing to sponsor a mere friend.

Should I be forced to choose between my career and the person I love? To me, the answer was clear. I spent my meager personal savings on a one-way ticket to Chile. I put my career on hold and took a job teaching English as a second



"There are many paths to a bright future."

language. I eventually found a job as a high school science teacher. After only a year of teaching and curriculum development, I was promoted to the school's director of science education. I collaborated with my partner, who was working at a local engineering university, to set up hands-on summer research experiences for my students who were interested in pursuing science, technology, engineering, and mathematics careers in college.

To make sure I didn't drop off my former adviser's radar, I published the research we had produced during my time in Puerto Rico. I lacked the computing power to analyze some of our genomic data, so I collaborated with bioinformaticians.

The U.S. Supreme Court struck down DOMA on 26 June 2013. I proposed marriage the same day. We were married less than a month later, in Argentina. We applied for a green card for my husband. I applied to graduate schools in the United States.

Earlier this year, I accepted an offer from the Division of Biology and Biomedical Sciences at Washington University in St. Louis, where I am currently rotating in some of the most influential immunology labs in the world. In April, I was awarded a National Science Foundation Graduate Research Fellowship. We are still pushing papers through the bureaucratic U.S. immigration system, but now we have a foundation of civil rights on which to stand.

Unexpected detours can improve skills and strengthen one's resolve to tackle science and society's challenging questions. There are many paths to a bright future. ■

David Anderson is a Ph.D. student and NSF Graduate Research Fellow at Washington University in St. Louis.

For more on life and careers, visit www.sciencereers.org.

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